Universidade do Minho
Escola de Medicina

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Cartilage Repair: the role of a scaffold in the repair of a cartilage lesion

agosto de 2018

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Tese de Doutoramento em Medicina

Trabalho efetuado sob a orientação do
Professor Doutor João Duarte Coelho Sameiro Espregueira- Mendes
e do
Professor Doutor Rui L. Reis

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DECLARAÇÃO

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STATEMENT OF INTEGRITY

I hereby declare having conducted my thesis with integrity. I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, August 1th 2018

Full name: Carlos Alberto Vilela Gomes

Signature: 

À minha esposa (Fernanda) e aos meus filhos (Carlos e Ana)

À minha família

Aos meus Amigos

Pelo tempo que lhes roubei e pela força que me deram

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Cartilage Repair: The Role of a Scaffold in the Repair of a Cartilage Lesion

ABSTRACT

Long-lasting repair of articular cartilage lesions remains a clinical unmet need, despite the multiple distinct approaches clinically implemented in the last decades.

Under this thesis, a thorough literature review and analysis was performed in order to understand what the current clinical and scientific practices are, and what are their main reported benefits and limitations, towards identifying new ways to tackle the current limited repair of cartilage lesions.

The field of tissue engineering and regenerative medicine has delivered extraordinary findings in subjects such as cell biology, material science, biochemical and biomechanical cues and animal models, allowing the development of innovative and sophisticated solutions, particularly for cartilage repair.

When aiming the regeneration of a functional articular cartilage tissue, the presence of healthy chondrogenic cells, at a therapeutically relevant amount, exactly at lesion site, is considered paramount. Several advanced scaffolding systems and surgical approaches have been developed to deliver cells and sustain tissue growth, yet retention of cells in situ has been suboptimal.

Herein, the experimental work developed in this thesis explores the potential of a methacrylated gellan gum (GGMA) hydrogel to deliver and retain chondrogenic cells in lesion site, while providing 3D filling of lesion volume during development of the new chondral tissue.

In vitro studies showed that GGMA hydrogel at 2% w/V is a suitable scaffold for encapsulation of human chondrogenic cells, such as human chondrocytes or human adipose derived stromal/ stem cells. Cells were maintained viable up to 21 days at densities ranging from 5-10 M/mL and chondrogenic differentiation was demonstrated by high collagen type II over-expression concomitant with low collagen type I. Techniques such as RT-qPCR and immunohistochemistry (IHC) were used to assess chondrogenesis.

Such promising *in vitro* outcomes supported the *in vivo* performance testing in a rabbit model with an induced critical-size cartilage defect. Herein, autologous adipose derived stromal/stem cells (10 M/mL) were delivered within GGMA 2 % w/V hydrogel by injection into lesion site and allowed for regeneration for 8 weeks. Histological analysis of tissue explants demonstrated new tissue

composed by hyaline-like cartilage (stained by safranin O) and collagen type II (identified by IHC). These histological results classified by O'Driscoll scoring were superior than those obtained for lesions treated by the gold-standard microfracture procedure ($p<0.05$) as well as for the untreated lesions ($p<0.001$). The gelification characteristics of the GGMA was compatible with an injectable system, which allows its application through the currently well-stablished minimally invasive arthroscopic procedures.

Given this, a new surgical tool was developed to allow the hydrogel delivery inside the joint under a standard arthroscopic approach. The device was effective to deliver the methacrylated gellan gum hydrogel directly into the chondral lesion created in a cadaveric joint. The hydrogel was maintained isolated of the liquid arthroscopic environment during gelification time (approximately 5 minutes), avoiding dilution/ dispersion of the hydrogel within the articular cavity. Hydrogel was maintained in lesion site after removal of the device form the joint. The flexible design of the surgical tool allows adoption of additional features and application in distinct settings if further explored.

The positive outcomes obtained under this thesis open an exciting route towards more efficacious and less invasive treatment procedure for cartilage repair, which is expected to increase cost effectiveness as compared to current treatment standards.

Tratamento da lesão da cartilagem: o papel de um scaffold no tratamento da lesão de cartilagem

RESUMO

Apesar das abordagens terapêuticas existentes, as lesões da cartilagem persistem sem uma solução clinicamente satisfatória a longo prazo.

Nesta tese é realizada uma profunda revisão e análise bibliográfica das práticas clínicas e científicas correntes, de modo a compreender as suas limitações e explorar novas formas de abordar a lesão da cartilagem.

No campo da engenharia de tecidos e medicina regenerativa têm-se testemunhado avanços extraordinários, particularmente em biologia celular e nas ciências dos materiais, no desenvolvimento de modelos animais, mas também na compreensão dos efeitos bioquímicos e biomecânicos, permitindo o desenvolvimento de soluções inovadoras e sofisticadas, especialmente na área da reparação da cartilagem.

Quando se ambiciona a regeneração funcional do tecido cartilagíneo, torna-se fundamental garantir a presença de células condrogénicas localizadas com precisão na zona pretendida, e na quantidade terapêutica adequada. Vários sistemas de administração celular *in vivo* têm sido desenvolvidos, juntamente com estruturas de suporte (*scaffolds)* ao crescimento celular e tecidular, contudo a retenção destas células no local da lesão durante o tempo necessário à regeneração persiste como sendo sub-óptima.

O trabalho experimental desenvolvido no âmbito desta tese, permitiu explorar o potencial do hidrogel de goma gelana metacrilada (GGMA), como agente de entrega e suporte de células condrogénicas no local da lesão de cartilagem, preenchendo volumetricamente a lesão durante o crescimento de novo tecido condral.

Os estudos *in vitro* demonstram a adequabilidade do hidrogel de GGMA 2% p/V no encapsulamento de células condrogénicas humanas, incluindo condrócitos ou células estaminais derivadas do tecido adiposo. As células, encapsuladas a densidades de 5-10 M/mL, mantêm-se viáveis até 21 dias de cultura, demonstrando diferenciação condrogénica através da sobre-expressão de colagénio tipo II concomitante com a sub-expressão de colagénio tipo I. Técnicas como qRT-PCR e imunohistoquimica (IHC) foram utilizadas para estudar a condrogénese.

Os resultados promissores obtidos *in vitro* suportaram a realização do estudo de performance *in vivo,* onde lesões condrais (consideradas críticas) foram induzidas na zona da tróclea do coelho, sendo o modelo animal recomendado para tais estudos exploratórios. Neste trabalho, células estaminais autólogas derivadas do tecido adiposo (10 M/mL) foram administradas em GGMA no local da lesão, e a regeneração do tecido foi permitida durante 8 semanas. Os resultados histológicos dos explantes revelaram tecido reparado composto por cartilagem tipo hialina (coradas pela Safranina O) e colagénio tipo II (identificado por IHC).

Estes resultados histológicos, classificados segundo a escala de O´Driscoll foram superiores aos obtidos com o tratamento standard, microfratura ($p>0,05$) assim como aos resultados obtidos nas lesões controlo não tratadas ($p<0,01$). As características de gelificação do GGMA foram compatíveis com um sistema injetável de administração do produto o que permite a sua aplicação num contexto de cirurgia minimamente invasiva.

Nesse contexto, um novo instrumento cirúrgico foi desenvolvido de forma a permitir a administração deste composto numa abordagem artroscópica clássica. O dispositivo foi capaz de realizar a administração direta do hidrogel na lesão de cartilagem que tinha sido previamente induzida num joelho de cadáver. O dispositivo permitiu também a manutenção desse hidrogel isolado do meio líquido da artroscopia, durante o tempo necessário para a gelificação do hidrogel no local da lesão (aproximadamente durante 5 minutos), impedindo a diluição / dispersão do hidrogel dentro da cavidade articular. O hidrogel foi mantido no local da lesão depois da retirada do dispositivo. O desenho do dispositivo permite ainda vir a desenvolver outras potencialidades no campo da artroscopia.

Os resultados obtidos no âmbito desta tese abrem novas perspetivas no tratamento mais eficaz e menos invasivo das lesões da cartilagem, fornecendo expectativas de uma solução terapêutica com melhor relação custo-benefício relativamente às opções atuais.

TABLE OF CONTENTS

LIST OF ABBREVIATION

A

AC	Articular cartilage
ACI	Autologous chondrocyte implantation
ACT	Autologous chondrocyte transplantation
AMI	Autologous matrix-induced chondrogenesis
AMIC	Autologous matrix-induced
aMSC	Adipose mesenchymal stem cells
ANI	National Innovation Agency
ANOVA	Analysis of variance
AP	Anteroposterior
ASC	Adipose-derived mesenchyma stromal/ stem cell
ASCs	Adipose-derived mesenchymal stromal/ stem cells
ASCs	Adipose mesenchymal stromal/stem cells
ASTM	American Society for Testing and Materials
ASTM	American Society for Testing and Materials

B

Beta-TCP	Beta-tricalcium phosphate
BMAC	Bone marrow aspirate concentrate
bmMSC	Bone-marrow mesenchymal stem cells
bmNC	Bone marrow nucleated cells
BMP	Bone morphogenic protein
bMSCs	Bone-marrow mesenchymal stem cells

C

C	Cells
CAIS	Cartilage autograft implantation system
cDNA	Complementary deoxyribonucleic acid
CE	Cell encapsulation
CTGF	Connective tissue growth factor
dGEMERIC	Delayd Gadolinium-enhanced magnetic resonance imaging of cartilage
DMEM	Dulbecco modified Eagle's minimal essential médium

E

ECM	Extracellular matrix
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
ETO	Ethylene oxide

F

FACS	Flow cytometry analysis
FBS	Fetal Bovine Serum
FDA	Food Drug Administration
FGF	Fibroblast growth factor
FLASH	Fast low-angle shot
FLASH	Fast low-angle shot
FSE	Fast spin-echo

G

GAG	Glycosaminoglycan
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde 3-phosphate
Gd-DTPA 2	gadolinium+ DTPA (diethylenetriaminepentacetate)
GDF	Growth differentiation factor
GF	Growth factors
GG	Gellan gum
GGc	Commercial gellan gum
GGMA	Methacrylated gellan gum
GGMA+ rASC	Methacrylated gellan gum + Rabbit adipose derived mesenchymal stromal / stem cells
GGp	Purified gellan gum

H

H	Hour
H_2O_2	Hydrogen peroxide
hACS	Human adipose-derived mesenchymal stromal stem cells/
hAT	Human adipose tissue
HMSC	Human mesenchymal stromal /stem cells
hNC	Human nasal chondrocytes
HSD	Honestly Significant Difference

I

ICRS	International Cartilage Repair Society
ICVS	Life and Health Sciences Research Institute
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IKDC	International Knee Documentation Committee
Kg	Kilogram
KOOS	Knee injury and osteoarthritis outcome
kpa	Kilopascal

M

MACI	Matrix-assisted autologous chondrocyte implantation
MDSCs	Muscle derived stem cells
MEM	minimal essential medium
MF	Microfracture
MFX	Microfractures
mg	Milligram

min	Minute
mL	Milliliter
Mm	Millimeter
MmHg	Millimeter of mercury
mMSC	Muscle mesenchymal stem cells
MOPS	Missouri Osteochondral Allograft Preservation System
MP	Mosaicplasty
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MSC	Mesenchymal stromal/ stem cell
MSCs	Mesenchymal stromal/ stem cells
MWCO	Molecular weight cut-off

N

NaOH	Sodium hydroxide
NC	human chondrocytes
NELL-1	Nel-like molecule-1

O

OA	Osteoarthritis
OAG	Osteochondral allograft
OAT	Osteochondral autograft transfer
OC	Osteochondral
OCD	Osteochondritis dissecans
OCT	Optical coherence tomography
OPF	Oligo(poly(ethylene glycol)fumarate)

P

PACI	Autologous chondrocyte implantation + periosteal flap
PBC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PGs	Proteoglycans
PLA	Polylactic acid
PLCL	Poly(L-lactide-*co*-3-caprolactone)
PLGA	Poly(lactic-*co*-glycolic acid)
PoC	Proof-of-concept
PRP	Platelet-rich plasma

Q

qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction

R

rASC	Rabbit adipose derived mesenchymal stromal / stem cells
RFE	Radiofrequency energy
RMI	Magnetic resonance imaging
RNA	Ribonucleic acid
RT	Room Temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

S

SD	Standard Deviation
SE	Spin-echo
Si-HPMC	silanized-hydroxypropyl methylcellulose
sMSC	Synovium mesenchymal stem cells
SNR	Signal-to-noise ratio
SPACE	3D fast SE sampling perfection
SPGR	Spoiled gradient-recalled echo
SSCs	Synovial stem cells
SSFP	balanced steady state free precession
SVF	Stromal vascular fraction

T

T	Tesla
TE	Tissue engineering
TETEC	Tissue Engineering Technologies
TGF-β	Transforming growth factor-beta
UK	United Kindom
USA	United States of America
w/v	Weight per volume
α-CD-EG	α-cyclodextrin/poly(ethylene glycol)
μ-CT	Microcomputed tomography
^{0}C	Degree Celsius
2D	Two dimensions
2D-FSE	2D Fast spin-echo
3Bs	Research Group in Biomaterials, Biodegradables and Biomimetics
3D	Three dimensions
3D-Bssfp	3D Balanced steady state free precession
3D-DEFT	3D Driven equilibrium fourier transform
3D-DESS	3D dual-echo steady state
3D-FSE	3D Fast spin-echo

LIST OF FIGURES AND TABLES

THESIS PLANNING

The present thesis is structured in 4 sections and 8 chapters. The first section is composed of a chapter presenting the aims and objectives of this work. The second section is composed of three chapters gathering relevant bibliographic references and significant research done in the area of cartilage lesions. This section reviews the diagnosis, treatments as well as the research done in the area of cartilage repair using hydrogels. Section III details the experimental work performed, both on what regards performance evaluation of methacrylated gellan gum hydrogel and stem cells for cartilage repair, as well as the development of a device for its application in an arthroscopic surgery. The final section contains the general final discussion and major conclusions. A brief description of each section is summarized below.

Section I – Chapter I

Chapter I – Contains aims and objectives of the thesis. This initial chapter is a very brief introduction explaining the relevance of the cartilage lesion in terms of its epidemiological and clinical implications and explains the major objectives of the present thesis.

Section II – Chapter II-IV

Chapter II – Presents an overview of the current knowledge about cartilage lesions. The epidemiological, social, economical and clinical framework is reviewed. The current state-of-the-art in cartilage lesion treatment is discussed and the distinct treatment options and respective outcomes are reviewed.

Chapter III – This chapter contains a brief review of the clinical management of chondral and osteo-chondral lesions and explores the future clinical and treatment approach presenting series of current clinical trials and enumerate new treatments arriving into the market.

Chapter IV – This chapter contains a review of the *in vivo* studies using hydrogels for treating cartilage defects conducted during the last decade. Herein, cartilage tissue repair by a tissue engineering approach is discussed. Employed materials, animal models, results evaluation, limitations and conclusions of those studies are presented.

Section III – Chapter V to VI

Chapter V – Describes the experimental work carried out towards evaluating the potential of methacrylated gellan gum (GGMA) hydrogels to sustain chondrogenesis, both *in vitro* and *in vivo.* To this end, human chondrocytes and human adipose-derived mesenchymal stromal/ stem cells (ASC) were tested. Cell viability, chondrogenesis and deposition of chondrogenic extracellular matrix were assessed *in vitro*. Cartilage repair by GGMA+ASC was further evaluated in an induced focal chondral lesion in a rabbit model.

Chapter VI – Describes the development of a surgical device to deliver and apply hydrogels in a surgical arthroscopic context. A summarized description of the device, the principal features and technical potential of the device are presented. The use of the device during an arthroscopic procedure in a cadaveric knee model is described and the major results and conclusions are presented.

Section IV - Chapter VII

Chapter VIII – Presents a general discussion of all the work carried out in the scope of this thesis, conclusions, future perspectives and personal considerations. It highlights major considerations regarding the set of the studies presented in this thesis and emphasizes the interest for new studies and new approaches concerning the subject.

Annex - I

Authorship and Co-Authorship of scientific papers, publications, oral communications, posters and patents, in the field of cartilage repair.

SECTION I

CHAPTER I

Aims

Taking into consideration the clinical and epidemiologic relevance, cartilage lesions are a significant, unsettling and worrying issue[1-6]. Bearing in mind the economic perspective and considering also the population aging, cartilage lesions are a very threatening problem with exponential growing costs[1, 5, 7-10]. For the patient itself, cartilage lesions are a real threat causing suffering and progressive deterioration of patient quality of life and expectations[9, 11]. The early diagnosis and adequate treatment could be the key for a successful solution[11, 12]. The clinical examination and imaging findings could help to achieve an early diagnosis[12-14]. However, cartilage lesions treatment is still under debate[5, 10, 13-17]. An efficient one-step and minimally invasive treatment would be the hope for those patients afflicted by a cartilage disease[18]. Hydrogel scaffolds seem to be suitable for being delivered in a joint and support cartilage repair[18, 19].

The aims of this thesis are:

1- Present the clinical picture, current treatments and future possibilities for repair of chondral and osteo-chondral lesions.
2- Understand current state-of-the art regarding the use of gels and hydrogels for repair cartilage.
3- Assess the *in vitro* performance of a methacrylated gellan gum hydrogel to support cell viability and chondrogenesis.
4- Study the *in vivo* potential of the methacrylated gellan gum hydrogel seeded with chondrogenic cells to repair induced cartilage defects.
5- Develop a device to deliver hydrogel formulations *in situ*, i.e. into the articular chondral lesion by a minimally invasive approach.

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SECTION II

CHAPTER II

Clinical Management of Articular Cartilage Lesions

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ABSTRACT

Articular cartilage is extremely sensitive to traumatic lesions and natural repair is very limited. When regeneration occur the tissue found in the lesion site is mostly fibrocartilage with poor mechanical properties, rendering a poor long-term clinical outcome. Cartilage lesion is a common problem with an impressive clinical and economic impact. With a difficult diagnosis in an initial disease stage, the cartilage lesion can progress to osteoarthritis and, therefore, a prompt diagnosis and treatment is required. Clinical management of cartilage lesions is a very demanding issue and the treatment is dependent of the extension, depth, location, chronicity of the lesions, patient's conditions and patients' expectations as well as associated lesions. In the present chapter, we present the clinical findings and diagnosis methodology to identify a cartilage lesion in an early stage. Finally, we discuss the indications, contra-indications, advantages, disadvantages and treatment decision-making as well as the outcomes of the available therapeutic approaches.

Key words:

Cartilage

Cartilage repair

Microfracture

Mosaicplasty

ACI

MACI

MASI

Tissue engineering

INTRODUCTION

Articular cartilage is a smooth, contact interface that lines the surface of two articulating bones of a diarthrodial joint. At the femur condyle the cartilage thickness ranges from 1.4 to 3.5 mm while at tibial plateau it ranges from 1 to 6 mm [1]. Although so thin, the cartilage presents excellent mechanical properties: providing a low-friction interface for the gliding articular surface and is able to support and distribute to underlying subchondral bone very high compressive and repetitive loads during a lifetime that can reach for the knee, 1.2 megapascals in each step [2].

Without a vascular, neural or a lymphatic network and due to the lack of progenitor cells, the cartilage has a limited capacity for self-recover from a lesion and represent a very difficult challenge to the orthopedic surgeon. In fact, a cartilage lesion is frequent being found in 61-66% of patients submitted to an arthroscopy [3-6]. About 900,000 Americans are affected by a cartilage lesion each year and more than 200,000 surgical procedures are done to solve this problem annually [5]. According to McCormick et al., the mean annual incidence is 90 surgeries per 10,000 patients with an annual incidence growth of 5%[7].Commonly, progression of the cartilage lesion is the rule resulting in osteoarthritis at later stages [8-11]. Radiographic knee osteoarthritis was found in 53% of symptomatic and in 17% of asymptomatic patients most commonly involving the medial and femoro-patellar compartment of the knee[12]. Total knee replacement is a poor and sad solution, especially for patients under 50 years old. Therefore, clinical and economic impact of cartilage lesions are significant: it is estimated that about 10-15% of adults over 60 years old suffer from osteoarthritis with direct and indirect costs over $65 billion annually [13]. Early diagnosis and treatment of cartilage lesions may play an important role by avoiding osteoarthritis development, patient suffering and saving important economic resources [4, 10].

CLINICAL FINDINGS

The mechanism of injury evolves an acute high-energy force or a shear and torsional force acting repetitively on the superficial articular surface[14]. The patient's history can offer some clues to the cartilage lesion diagnosis and evaluation. How the complaints started, how long are they affecting the patient, which activities are pain provoker, what was the lesion mechanism, what is and was the normal activity of the patient, what are the real expectations of the patient for his future and return to his normal daily-life or sport activity. Those are questions to answer in order to reach a good evaluation of the pathology. Pain is the main patient symptom and its intensity is variable and described in many ways. Usually, pain worsens with activity[4], have a mechanical rhythm and are related to a previous trauma[4]. In other occasions, like in inflammatory or

degenerative disease, pain is not dependent of the physical activity. There is a very wide range of causes for complaints exacerbations in daily or sport activities. Swelling of the joint can also be present. For the knee, climbing or descending stairs, arising from a chair can cause pain. When a loose-body is in the joint due to a cartilage loose fragment, patients can refer symptoms like giving-way, locking and pseudo-locking. Sometimes patients have symptoms related to other pathology like a meniscal tear or a ligamentous injury [4, 15]. A careful and complete physical examination of the affected joint is required and the search for a swelling, hemarthroses, limitations of the range of joint motion, painful crepitation are mandatory, as are the specific tests for the examined joint. The examination is complete when compared with the contra-lateral joint.

Once symptomatic, the patient's pain and the functional impairment are likely to progress. During the course of this disease, the patient has some asymptomatic periods, but symptoms will return and worsen. Cartilage lesion evolution is not well known, but is believed that is dependent of the nature and type of lesions, associated lesions, patient gender, patient genetics and patient co-morbidities [10, 16]. Normally, when a cartilage lesion occurs, the repairing process produces a fibrocartilage mostly with type I collagen and abnormal proteoglycans without the mechanical properties of the normal articular cartilage and consequently more susceptible to breakdown and to an early osteoarthritis [17]. Initially, when an articular cartilage injury occurs, a macromolecules loss is observed followed by a cartilaginous matrix rupture and finally by rupture of the bone matrix. Those three cartilage lesion evolutions stages must be taken in consideration when the therapeutic approach is chosen[6]. Therefore, the extension, deepness, and location of the lesion are important characteristics to define how serious the lesion is. Associated lesions as meniscal tears or ligamentous injuries[10, 18-20], misalignment of the limbs[10, 21], obesity[10, 22-24], and previous failed treatments are others factors to take in account to a correct evaluation of the cartilage lesion severity.

Outerbridge in 1961 created a four-grade classification score to evaluate the macroscopic changes in cartilage lesions of the patella. In Grade 1, the less severe lesion, only softening and swelling of the cartilage is observed (figure 1). Grade 2 included cartilage lesions with fragmentation and fissuring in an area 1,5cm or less in diameter (figure 2). For Grade 3 the lesions presented also fragmentation and fissuring but involving an area more than 1,5cm in diameter. Grade 4, the most severe, included the lesions with erosion of cartilage down to bone (figure 3). Grade 1 and 2 are considered low grade lesions, but they do not heal and usually progress to a more severe stage [25]. This classification was adopted and became popular as a classification system for other

Figure 1 - *Cartilage softening (Grade 1)*

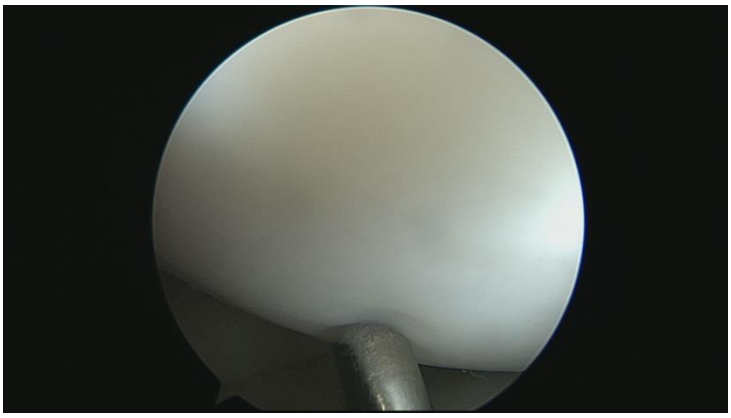


Figure 2 - *Cartilage fragmentation and cartilage fissuring (Grade 2)*

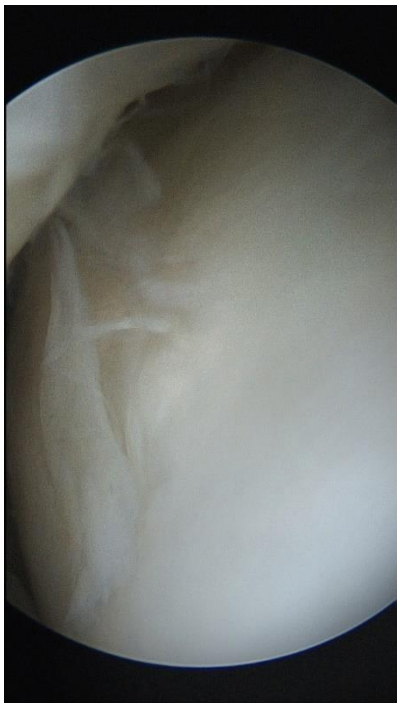
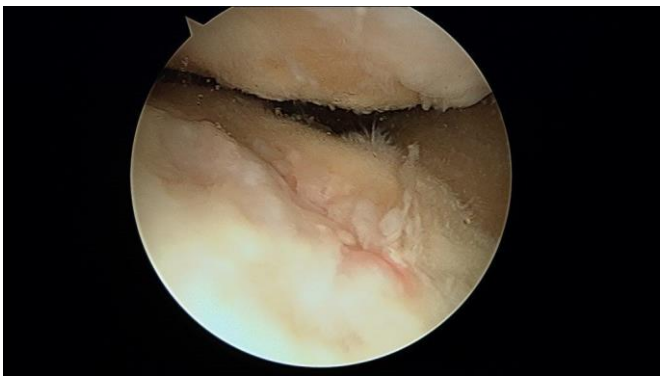


Figure 3 - *Cartilage erosion Grade (3)*



cartilage lesions in the knee and for other joints. The Outerbridge score allow the distinction between a partial (Grades 1 and 2) versus nearly full or full-thickness cartilage defect (Grades 3

and 4); between a small (Grade 2) and larger (Grade 3) lesion; and describes a complete loss of cartilage (Grade 4). However, the Outerbridge classification has specific limitations: for example, an extensive partial thickness defect with a potentially bad prognosis, due to its size, is a Grade 1 defect. Whereas a direct cut or narrow fissure is a Grade 4 defect [26].

The International Cartilage Repair Society (ICRS) created a modified classification system that focuses on the depth of the cartilage injury combined with visual measurement (ICRS Cartilage Injury Evaluation Package). The ICRS grading score is a five-grade score and intends a better macroscopic description of the defect and a better correlation with clinical outcome. Grade 0 relates to normal cartilage. In Grade1 are included superficial cartilage lesions, fissures, cracks and cartilage lesions with indentation (Grade 1A for the lesions with softening and/or superficial fissuring; Grade 1B when fissures and cracks were present. In Grade 2, fraying is found, lesions extending down to <50% of cartilage depth. In Grade 3 the cartilage lesions present a partial loss of cartilage thickness and cartilage defects extending down >50% of cartilage depth as well as down to the calcified layer. Grade 4 lesions relate to lesions with a complete loss of cartilage thickness and bone is visible [27].

Several other historical grading systems based in arthroscopy and/or MRI findings have been utilized: Insall (1976), Ficat et al. (1977), Casscells (1982), Beguin and Locker (1983), Bentley and Dowd (1984), Noyes and Stabler (1989), Frenche Society of Arthroscopy grading system (1994), Lewandrowski et al. (1996), Hunt et al. (2001) [18].

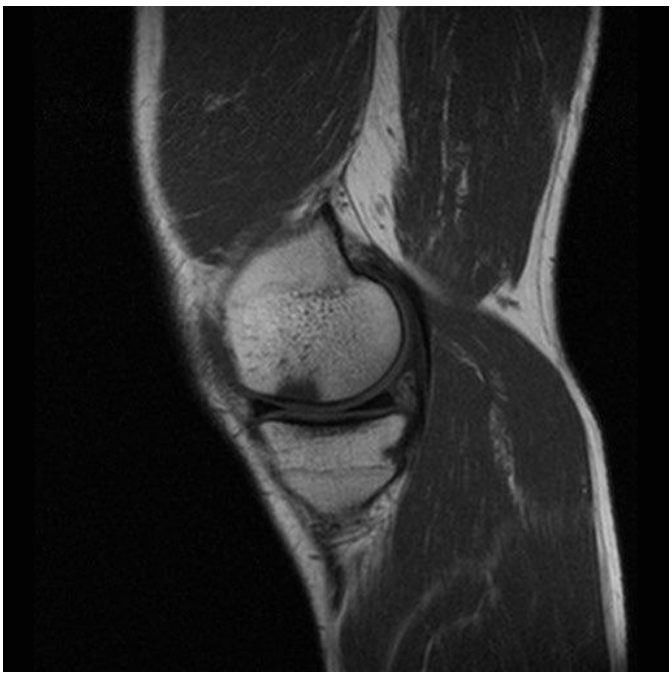
According to ICRS classification, Grade 3 was the most common lesion found with 55% of all patients submitted to an arthroscopy, followed by ICRS Grade 2 and ICRS grade 1 lesions and only in 5% of all patients presented an osteochondral grade 4 lesion [3, 28]. The majority of the cartilage lesions are single, affecting patients over 50 years old, with a mean area of 2,1 cm^2 (range between 0,5 and 12 cm^2), related to a previous trauma and affecting more frequently the medial condyle (in 58% of the cases). Patella was the second place more frequent to have a cartilage lesion with 11% of all the patients [3]. According the same authors, a concomitant meniscal, or ligamentous lesion was visible in 42% and 26%, respectively. Others authors found similar results [4, 15, 22, 28]. According to Aroen et al. arthroscopy review work, about 6% of the patients submitted to an arthroscopy have an ICRS grade 3 or 4 cartilage defect with a size over 2 cm^2 and 11% of all patients reviewed show cartilage defects suitable for cartilage repair procedures[4].

Most of the radiographic studies are normal and fail to reveal the majority of chondral lesions [29-31]. Although this evidence, radiographs can be very useful in patients with bigger osteochondral

lesion, in patients affected by a severe osteoarthritis, osteochondritis-dissecans or a limb malalignment. For the study of the affected joint, at least an anteroposterior (AP) view and a lateral view are required. In some cases, a more specific view can be helpful. For the evaluation of the knee, the radiographic protocol includes a anteroposterior view, a standing anteroposterior view and a lateral view with the knee flexed 35°. A patellar view to study the patella is mandatory [12]. If the AP standing view or the clinical evaluation reveal a deformity in varus or in valgus a full-length standing radiograph can be useful. Rosemberg et al. concluded that a major chondral lesion was present when a narrowing of the joint of more than 2mm compared to contralateral knee space in a 45° posteroanterior weight-bearing view was visible [32, 33].

Magnetic resonance imaging (MRI) is the most appealing, powerful and important diagnostic procedure for the evaluation of cartilage lesions (figure 4). MRI is a noninvasive procedure and provides a more accurate information than radiographic studies and can document chondral lesions prior to radiographic changes and even prior to arthroscopy [34, 35]. In fact, MRI can detect metabolic and structural defects including the water content, before noticed in an arthroscopy [34]. Thus MRI is very useful for cartilage lesions diagnosis [35-38], for monitoring the effects of chondral pharmacologic and surgical therapies, for study the cartilage disease evolution and in cartilage scientific research, namely in the semiquantitative and quantitative assessments of cartilage [34, 35]. However, for proposal therapies, although operator dependent, arthroscopy is yet the gold standard and the elected diagnose and validation procedure [34, 36].

Figure 4 - *Cartilage lesion - RMI image*



For chondral diagnosis a High-magnetic-field-strength 1,5-3,0 tesla (T) scanner is needed (figure 5), which provides a higher signal-to-noise and contrast-to-noise ratios and, therefore, a thinner slice and a higher space resolution imaging. A minimum magnetic-field-strength of 1,0-T is needed for morphologic assessment of knee cartilage, but 1,5-T is currently used in cartilage evaluation. With a magnetic-field-strength of 3,0-T the time imaging acquisition is reduced, the image quality and image resolution is improved and, therefore the diagnose accuracy [39, 40]. However, the use of a higher strength magnetic field improves the magnetic susceptibility and the deposited energy in the tissues, images are more vulnerable to flow artifacts and the severity of chemical shift effects increases [41]. The 7,0-T MRI protocols was used in few studies and have not yet clearly shown advantages when compared with 3,0-T protocols [41, 42].

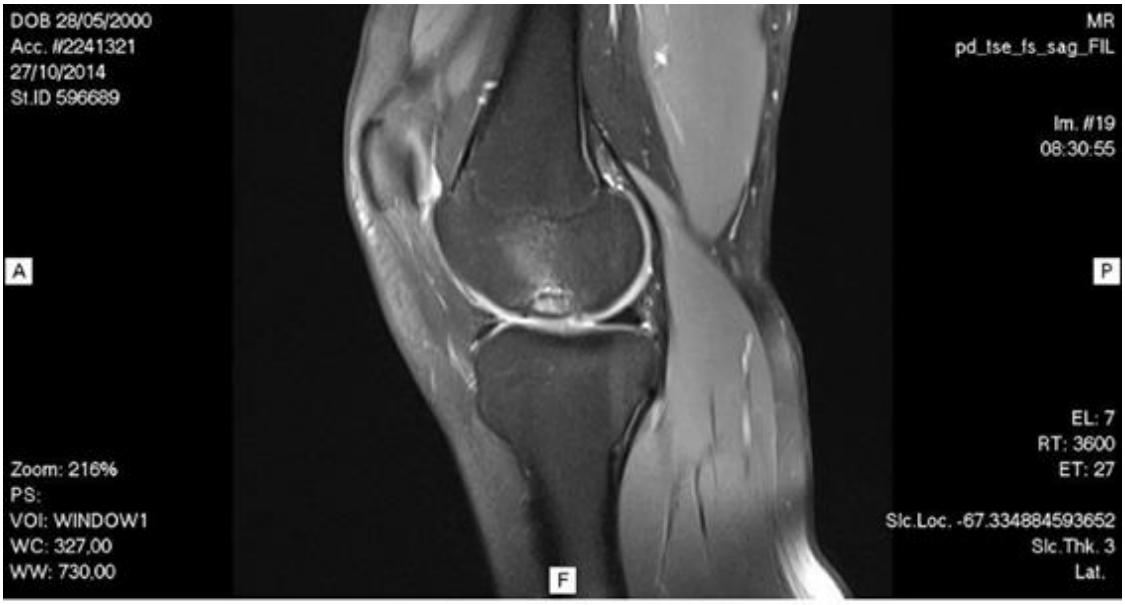


Figure 5 *- Cartilage lesion - RMI image*

A voxel is a rectangular volume element of the MRI images. The signal intensity of the voxel is a proportional sum of the signal of the composing tissues and the manipulation of the intensity of the contrast allows the highlight different tissue types. When an image is composed for two different tissues an artifact can occur in the interface of those tissues and is the reason for an incorrect evaluation of the cartilage lesions like the lesion dimension and cartilage thickness or even the diagnosis of a cartilage defect.

MRI imaging provides a morphologic characterization of the cartilage lesions and defines the deepness and extension of the lesions. Several acquisition techniques have been proposed: 2D and 3D fast spin-echo (FSE), 3D spoiled gradient–recalled echo (SPGR), 3D driven equilibrium fourier transform (3D-DEFT), 3D dual-echo steady state (3D-DESS), 3D balanced steady state free

precession (3D-bSSFP), 3D fast SE sampling perfection with application-optimized contrast using different flip-angle evolutions (SPACE)[19].

2D-FSE is the most commonly used in the assessment of the joint cartilage and allows a good diagnosis of bone, menisci, or ligamentous injuries. For cartilage lesions, a good correlation and high sensitivity and specificity with arthroscopic technique was found [43]. 2D-FSE is recommended by the International Cartilage repair society for the evaluation of cartilage repair. 3D-FSE can reduce the time acquisition and has a diagnostic performance similar to 2D-FSE techniques but has not yet replaced the 2D-FSE in clinical practice [19, 44] .

3D-SPGR is the gold standard technique for morphological knee cartilage evaluation. 3D-SPGR is very sensitive with a high accuracy to detect cartilage lesions and is very useful for cartilage thickness and volume measurements. Although those advantages, SPGR fails in the diagnosis of bone, menisci or ligamentous associated lesions. Besides, long time imaging is required and more metal artifacts are related [19, 44, 45]. Fast low-angle shot (FLASH) imaging is an SPGR technique useful for assessment of knee cartilage repair [41, 44, 46].

3D-DEFT increases contrast between fluid and cartilage and preserve the cartilage signal, resulting in a high signal intensity in both cartilage and synovial liquid [41, 45] but has a long acquisition times with a consequent vulnerability to motion artifacts. 3D-DEFT has a comparable performance to detect cartilage lesions when compared with FSE and SPGR techniques and is not reliable for assessing bone marrow [41, 44]. 3D-DESS has a shorter acquisition time than SPGR with similar accuracy for the detection of cartilaginous lesions. 3D-DESS allows a quantitative assessment of cartilage and decreased volume artifacts and has been validated for clinical use[41, 45]. 3D-bSSFP provides a good synovial fluid-cartilage contrast, decreased volume artifacts and is eventually useful for the study of other structures of the knee. VIPR Imaging is a SSFP derivative with shorter acquisition times and probably interesting for clinical and research practice[41, 45]. SPACE although the long acquisition times, has a good signal-to-noise ratio (SNR) and a high SNR efficiency but the capability to distinguishing cartilage and surrounding tissues is not as good as others techniques [41].

MRI provides also a compositional imaging of cartilage. The properties of the collagen and proteoglycan–associated glycosaminoglycan's macromolecular network of the hyaline cartilage, its content, electric charge, and status are assessed by MR imaging techniques. The current techniques that are available for the assessment of cartilage are focused on collagen and glycosaminoglycan content and include: T2 Mapping, dGEMERIC, T1p imaging, sodium imaging,

and diffusion-weighted imaging [41, 44]. T2 Mapping reflects the interaction between the water content and the collagen network in a grey-scale map or in a color map and identifies the early stages of cartilage degeneration and the treatment effectiveness over time [41, 47]. T2 Mapping is clinically useful, well validated, but is a 2D- technique with a long acquisition time. dGEMERIC is related to the concentration of the negatively charged glycosaminoglycan molecules. The dGEMERIC acquisition RMI technique is well validated and clinically useful, but requires the administration of an intravenous contrast product and has a long acquisition time [41, 44]. The intravenous administration of Gd-DTPA 2- and is consequent and progressive concentration in cartilage is inversely proportional to the glycosaminoglycan content and is an evaluation method for monitoring cartilage repair procedures [41]. T1p Imaging is dependent of collagen network and glycosaminoglycan content and higher T1p Imaging values are indicative of a damaged cartilage. The use of T1p Imaging is limited to a few research centers and time consuming, thus limited for clinical use [41, 44]. Sodium imaging MRI acquisition technique is related to the glycosaminoglycan composition of the cartilage and can be useful in the differentiation of early stage of cartilage pathology. Sodium imaging MRI is available in few centers and need a special hardware. Diffusion-weighted imaging is based on the motion of water content which is related to the cartilage architecture and cartilage biochemical structure, thus dependent of the collagen and glycosaminoglycan content. This technique can be useful for the implants follow up [41].

With a FSE acquisition technique the lesion appears to be brighter than adjacent cartilage but with an SPGR acquisition technique the lesion is darker than adjacent normal cartilage [44]. The estimated area, depth, the presence and the volume of the bone attached to the cartilage defect, the exact location of the defect and edema bone marrow signal should be reported and crucial for the treatment plan and can provide some prognosis hints. MRI is progressing and becoming a more sensitive and specific diagnosis procedure. MRI is also a more common monitoring cartilage progress repair procedure [17, 44].

TREATMENT

Surgical cartilage lesions treatment has a long history and very early attempts to treat cartilage were described in the last century. One of the first osteoarticular transplants was described in 1925 and most of the current marrow stimulation procedures derived from initial studies of Pridie(1959) and Ficat (1979)[5].

The goals of cartilage repair are to diminish pain and swelling in afflicted patients; improve function and sports activities; prevent progression towards osteoarthritis; achieve these goals with lowest cost to society and lowest co-morbidity possible to the patient. For the clinicians' decision in cartilage repair, is important the characteristics of the lesion and etiology, the defect thickness, location and size. It is also important the containment, a ligamentous or meniscus injury, previous treatment, physiologic age and systemic disease. Therefore, the treatment must be focused on the specific patient we want to treat and there is no consensus regarding the best method to repair a cartilage defect [48] and about the long-term results [49]. The choice of the best treatment for our patient demands more rigorous prospective, adequately powered and randomized clinical trials. Besides, there are no long-term studies comparing the treated lesion with the untreated lesion which means that the cost-benefit ratio is yet unknown[6].

Some factors are related to a better clinical treatment result: age under 30 years, body mass index <30, a correct limb alignment and integrity of menisci or ligamentous. Lesions in the medial femoral condyle have better results than lesions in the lateral condyle, tibia plateau or in the patella[6].

Palliative treatments

The articular lavage / debridement using a saline solution inserted into a joint with a needle or during an arthroscopy associated with the removal of chondral fragments and osteophytes, lose bodies, degenerated menisci and redundant synovia [50] is an empirical therapeutic approach without a solid scientific or biological basis for the symptomatic beneficial effects reported in few reported studies. Besides, the relative pain and symptoms relief is not consensual and some authors did not found this supposed clinical improvement with the articular lavage [51, 52]. This procedure is in decline [51, 52] and the indications are, eventually, limited to a patient with locking symptoms due to a loose body, in cases of unstable cartilage or with a concurrent meniscal tear [6].

Electrocautery, laser or radiofrequency energy (RFE) devices have been used for the treatment of cartilage lesions. In the electrocautery procedure, the tissue electrical tissue resistance to a high-frequency current, produce tissue destruction. Various devices are available, and the results could not be better than simple chondroplasty. Laser was introduced to arthroscopy surgery in the 1990's and the effects produced when the laser energy touches a tissue are reflection, scatter, absorption and transmission. Absorption is the predominant effect that causes tissue heating. RFE systems

for clinical application can be monopolar or bipolar and the experimental and clinical reported results are controversial and contradictory [53-55]. RFE is relatively inexpensive, safe and simple to use in an arthroscopic surgery and almost replaced the laser and electrocautery procedures for thermal chondroplasty. Various systems are available and under development: Vulcan EAS TM, Linvatec, VAPR TM, ArthroCareTM, UltrAblator Electrode. Although the clinical outcome regarding the use of RFE in cartilage lesions treatment are few, encouraging results have been reported with significant pain-relief but concerns about costs and security related to osteonecrosis, cartilage loss, proteoglycan loss and avascular necrosis limits its use [54, 56, 57].

Reparative treatments

Stimulating bone marrow techniques include arthroscopic abrasion arthroplasty or simple abrasion chondroplasty, Pridie drilling and microfracture (MF) techniques popularized by Steadman at al. Spongialization is also a stimulating bone marrow procedure adopted for patellar lesions described by Ficat and colleagues. The rationale behind this concept is to stimulate a spontaneous and natural repair reaction by penetrating the subchondral bone and consequent spongeous bleeding with the resulting blood clot, promoting the recruitment of bone marrow cells to enhance a natural healing [58]. The usual repaired tissue is not a normal hyaline cartilage [58, 59].

Arthroscopic abrasion arthroplasty or simple abrasion chondroplasty is a salvage and palliative arthroscopic debridement procedure. Using a motorized burr or a curette the surgeon removes the superficial dead bone of the cartilage lesion, exposing viable bleeding bone, and leaving untouched the normal surrounding cartilage. The major clinical indication is the advanced and extended grade III/IV lesion or in an severe degenerative arthritis in an older patient, usually around 60 years old who is seeking for an alternative to a total knee arthroplasty. The surgery has been used for more than 25 years and the results are controversial. Some authors reported a clinical improvement [60] and a deferred knee joint arthroplasty for more than 5 years with a long durability of the repaired tissue. Other studies did not find this clinical improvement and concluded that arthroscopic results are no better than medical treatments or even placebo treatments [61, 62]. Although this lack of scientific evidence, chondroplasty and debridement procedures are the most performed procedure in the United States [7].

MF technique, as described by Steadman et al., included the complete cartilage lesion identification, debridement of all remaining cartilage fragments till the healthy cartilage limit creating a vertical stabilized shoulder (figure 6). The calcified cartilage layer of this well delimited

area is removed. Using a small Awl and a mallet, perpendicular holes to the subchondral bone of 3-4 mm depth separated by 3-4mm are done [63]. A specific rehabilitation program is required and usually a variable non-weight bearing period is demanded. This procedure become popular and widely used as a cartilage restoration procedure [5] and comparative technique with all other techniques[6]. Despite a good short-time clinical outcome[58, 59, 63], the repaired tissue is not hyaline cartilage but a fibrocartilage with poor mechanical properties [64, 65] and the results of few long-term studies following microfracture treatment for cartilage lesions are not conclusive [17, 58, 59, 66]. According to McCormick et al., MF and drilling are the second restorative cartilage procedure more often performed in the United Sates[7] and good clinical and imaging results were reported[67] even when compared with other procedures more expensive and demanding like mosaicplasty or autologous chondrocyte implantation (ACI) [48, 59]. The indications are still under debate. The benefits of this technique according the location of the lesion, patient´s age and the upper lesion size limit is not clear and controversial results were reported. According to Steadman [63] an improved outcome is expected in all knee compartments in patients with cartilage lesions greater than 4 cm^2 in patients under 45 years old and even better, under the age of 35 years old [63]. Other authors did not find the same good results in lesions greater than 4 cm^2 or in patella chondral lesions [5]. Goyal et al. in a systematic review observed that MF in patients with a small lesion and low activity had a good short-term outcome and beyond 5 years post-operatively a failure of the treatment could be expected[68]. Chondral lesions with subchondral bone intact with an area lesion lesser than 2-2,5 cm^2 [11, 58, 69, 70] in a patient younger than 35 years old with a body mass index under 25 Kg/m^2 and a knee cartilage lesion with no more than 12 months evolution appeared to be the best indication for the microfracture technique[6, 70].

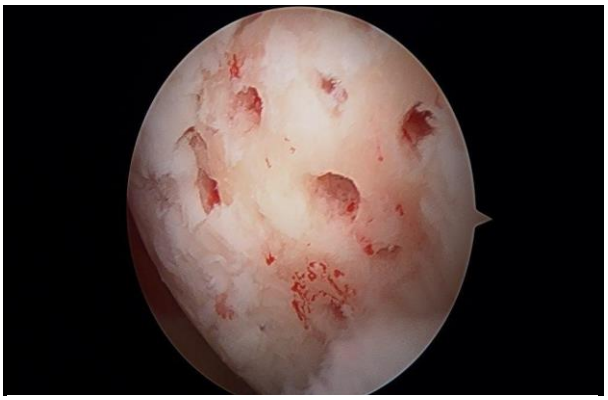


Figure 6 - *Cartilage treatment according to the MF technique*

The association of those techniques with growth factors, platelet-rich plasma (PRP) or genetic engineering have been studied and may provide in the future an alternative and an improvement in cartilage treatments[71-73]. Another approach is the AMIC (autologous matrix-induced chondrogenesis) technique consisting of covering a cartilage lesion initially treated with MF with a

collagen (I/III) membrane. This technique can be associated with the application of PRP or concentrated bone marrow (BMAC - bone marrow aspirate concentrate). The published results showed a clinical improvement, but the filling of the defect in the MRI analysis is not conclusive [74-76].

Dr. Craig Morgan, Dr. Vladimir Bobic and Dr. Lazlo Hangody popularized the osteochondral autograft transfer technique[5]. Osteochondral autograft transfer (OAT) is an alternative cartilage procedure: harvesting a cartilage autologous plug from a non-weight bearing area of the knee or from other joint [77] to repair the cartilage defect (figure7). In the knee, the most frequent donor site is the medial and lateral border of the condyles, the intercondylar notch or the sulcus terminalis of the femoral chondyle [58, 78]. Useful for symptomatic small chondral or osteochondral defect, between 2,5 cm and 4cm^2 in the weight bearing of a young patient [78-80] and has a better outcome in lesions located in the condyles than in patella or tibial plateau [6].

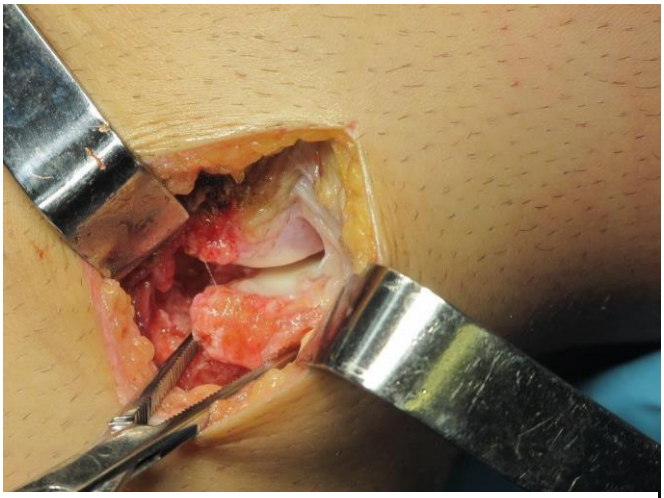


Figure 7 - *Cartilage harvessting from tibio-fibular proximal joint*

In an ACI or MF failed procedure, OAT can be an alternative treatment [78]. The OAT is a surgical demanding procedure and has other limitations: morbidity of the donor site and limitations of the available graft, congruency of the repaired surface specially when is necessary more than one plug to fit a more extensive lesion in a technique so called mosaicplasty (figure 8) [79, 80]. The osteochondral relocated plug has a good bone-to-bone potential healing, but rarely heals completely with the surrounding healthy cartilage. Good results have been reported [6, 79, 81, 82] even when compared to debridement and MF techniques [83, 84]. Solheim et al. reported a poor outcome or even failure of the mosaicplasty in 40% of the patients in a long–term follow up [85]. The best results with mosaicplasty technique were reported in a small deep lesion under 2,5 cm^2 located in the medial condyle[74] an CAIS - Depuy-Mitek, Raynham MA.

A frozen or fresh osteochondral allograft can be the treatment of choice for a patient aged up to 50 years and /or active patient with an extensive chondral or osteochondral lesion, usually greater than 2,5 cm and when arthroplasty is the alternative [6, 78]. Other indications for this procedure include salvage of previous cartilage procedure, osteochondritis dissecans, osteonecrosis of the

femoral condyle or a reconstructive post-traumatic surgery [78]. The surgical technique is demanding to achieve a good fixation and congruency with the healthy adjacent bone and cartilage. Medico-legal reasons, the risk of potential disease transmission, the low availability and the difficulties in preserving and managing the allografts, are serious drawbacks limiting the choice of this treatment. Immunogenicity of the allograft is also limiting and a percentage of patients become antibody positive with a less favorable outcome when compared with antibody negative patients [5]. Good clinical outcomes in medium / long-term have been reported [5, 86] but others studies did not confirm these results and reported a high reoperation and failure rate [87].

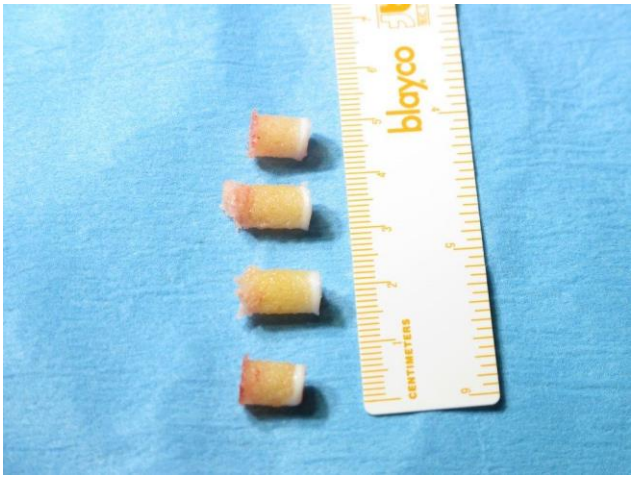


Figure 8 - *Prepared osteochondral grafts before the application in mosaicplasty*

Allogenic cartilage grafts are a recent therapy for cartilage lesions with the advantage of a lower immunological response. A morselized cartilage allograft or an allogenic chondrocyte implant is available for cartilage repair (DeNovo® NT – Zimmer, Warshaw, Indiana). The surgical technique is similar to the MF/ Autologous chondrocyte implantation (ACI) and clinical improvement were reported in a few studies already reported [5, 78]. The cartilage autograft implantation system (CAIS - Depuy-Mitek, Raynham MA) is a single-stage procedure utilizing a glued autologous morselized cartilage onto a synthetic bio absorbable scaffold instead an allogenic graft.

Acellular three-dimensional scaffolds made up of more than one layer to mimic normal cartilage structure, have been proposed as cartilage regenerative procedure. The rational of these techniques is to provide a structural support for immature reparative tissue resulting from the bone marrow stimulation. The simplicity of the procedure and the possibility of combination with cells or growth factors, make this technique an interesting approach. However, pain and persistent swelling have been reported [6]. TruFit® (Smith & Nephew,Andover,MA) is a bilayer porous PLGA-calcium-sulfate biopolymer that was proposed for cartilage regeneration. The early reports were good but the repaired tissue seemed to be heterogeneous, with cyst formation in the subchondral bone and any evidence of bone ingrowth, osteoconductivity, or integration. Unfavorable mid-term MRI results were reported[75]. The commercialization of this scaffold was suspended. Maioregen® (Fin-Ceramica S.p.A., Faenza, Italy) is a nanostructured biomimetic hydroxyapatite-collagen

scaffold with a porous 3-D tri-layer composite structure available for clinical use. Initial good clinical results were re-ported, but the follow-up is short and the studies are few [74, 75, 88].

Regenerative treatments

Autologous chondrocyte implantation (ACI) / Autologous chondrocyte transplantation (ACT) is a regenerative two-step cartilage therapy introduced in Sweden in the late 1980's by Peterson and Brittberg to resurface a symptomatic patient with a cartilage lesion. The first step is the assessment of the joint and performance of a cartilage biopsy. The procedure begins with the cartilage harvesting of approximately 200-300 mg of articular cartilage from a healthy and non-bearing area of the donor. The harvested cartilage fragment is processed to achieve chondrocyte isolation and expansion to a high chondrocyte density, usually between 5 and10 million cells over a period of 4-6 weeks [17]. In the second step, a periosteal flap, harvested from the proximal tibia and 2mm larger than the lesion, is sutured to the healthy borders of the prepared, non-bleeding and clean cartilage lesion. The covered lesion is than sealed with glue usually collagen or hyaluronan secured with fibrin glue or is self-adhering. Finally, expanded chondrocytes are implanted into the closed lesion. [6, 14, 75].

The cartilage defect coverage in ACI first generation (ACI – 1st generation) is made with a periosteal flap. For the ACI second generation (ACI-2nd generation), a membrane made often of collagen type I/III is the choice to cover the defect. The pointed advantages are decreased surgical exposition, reduced operating time and reduction of the complications related to the periosteal use [14, 64] despite the reported asymptomatic graft hypertrophy [64] . In ACI third generation (ACI-3rd generation) a matrix is seeded with cells and implanted in the cartilage lesion, the ACI-3rd generation is so called MACI (matrix-assisted chondrocyte implantation), but this was adopted as a trademark of Genzyme Biosurgery [11]. These treatments use a chondroinductuive or chondroconductive matrix usually seeded with autologous cells in controlled conditions to improve mechanical properties before the surgery. It is believed that ACI-3rd generation has an even chondrocyte distribution and there is no need of sutures or either a coverage which reduces the time of the surgery and the surgical exposure [14].

The indications for a ACI/ACT treatment in a knee cartilage lesion are well motivated patients under 55 years old, with pain, swelling, locking or catching with a grade II or IV cartilage lesion. ACI has been used to restore focal defects between 2-12cm^2. However, it has been used in lesions up to 26,6 cm^2. In defects under 2 cm^2, ACI is indicated as a salvage procedure with poor reported

outcomes. The best location is the femoral or patellar articular surface without a kissing lesion in the opposite articular surface. ACI is contra-indicated in patients with an inflammatory arthritis or with an articular infection associated lesions described above must be considered and included in the treatment plan[6, 11, 14, 17]. For the talus an ACI treatment is recommended in patients with a lesion greater than 2,5cm in diameter and as alternative autograft or allograft transplantation could be chosen as an option[87].

Complications for the ACI 1st generation are related to the periosteal flap: the graft and/or periosteal delamination and periosteal hypertrophy were related. Technical difficulties, large exposition and stiffness of the joint are also drawbacks of this technique due to the large tissue exposition [11, 14]. Other complications have ben also reported: device rejection and migration, immune reaction, delamination, swelling, fever and joint stiffness [76, 79] Better results were reported with ACI-2nd generation in a systematic review [69, 85].

ACI become a popular technique treatment to repair cartilage lesions and has been performed on an estimated 35,000 patients worldwide [17]. The bibliography review of ACI – 1st generation studies show an improved clinical, histological and mechanical results where, even in long-term follow up [6, 14, 17, 89]. good to excellent clinical outcomes were reported in studies with patients older than 45 years or in patients refractory to prior treatments [14]. A clinical improvement and lesser complications related to the periosteal graft was reported using type I/III collagen membranes [11, 90] but in other studies this finding was not confirmed [11]. Comparing ACI – 1st generation with a ACI-2nd generation and ACI-3rd generation the results are not conclusive but some reported results are better with ACI -3rd generation treatment [6, 65, 66]. Although the reported good results [75, 91, 92] it has not been possible the regeneration of hyaline cartilage in a consistently way [5, 11]. Comparing ACI procedures with others techniques (arthroscopic debridement, mosaicplasty) as Batty summarized, better significant clinical outcomes were found with ACI treatments[14, 17, 91, 93]. For the comparison of the ACI procedures with microfracture procedure, although a better clinical outcomes with ACI treatment, studies do not find always a statistically significant improvement [6, 14, 17, 64, 91, 93-95]. However, ACI was associated to a better structural repair, in more recent studies, a better long-term outcome was found with ACI 3rd generation [6, 14]. Although ACI 3rd generation has been used since 2000, few studies aimed to correlate the arthroscopic findings of a second-look arthroscopy with the histological appearance of the ACI biopsy [65]. As Enea et al. demonstrated a nearly normal cartilage appearance of the repaired tissue with an ACI 3rd generation in a second-look arthroscopy in 80% of cases and is not

related with the histological findings of hyaline cartilage in 20% of cases or with the functional patient´s status[65]. An important drawback of ACI techniques is the costing analysis as the Medical Service Advisory Committee of Australia reported. ACI procedures are more expensive than either MF or mosaicplasty due to the chondrocyte cell culture [91] but the cost-benefit over MF technique in terms of quality-adjusted life gained and osteoarthritis–related costs was also documented [64].

While chondrocytes are of great interest but due to its small initial number of cells and due to the de-differentiation on expansion to a fibroblast-like phenotype and consequent decreased proteoglycan synthesis and type II collagen expression and increased type I collagen expression, new alternatives cell sources are gaining increasing interest in the last years. Bone marrow stem cells (bMSCs), adipose stem cells (ASCs), Muscle derived stem cells (MDSCs), synovial stem cells (SSCs) and embryonic stem cells (ESCs) have been investigated in *in vivo* and pre-clinical studies [96, 97] but, to our knowledge, chondrocytes are the only cell source currently approved for clinical use. A great amount of cells that easily can be obtained without any adverse effect in the donor site, and the easy differentiation and expansion are pointed advantages for these alternative sources of cells as are the immunosuppressive and anti-inflammatory properties [93, 97, 98]. MSCs could be a suitable treatment option for cartilage repair and the few available data shows pre-clinical and clinical outcomes, at least, similar to the use of chondrocytes[99]. More and better quality studies and long-term follow up are needed to find the final conclusion [100]. Some questions need more affirmative answers: the optimal MSC source, the quality and durability of the repair tissue, the resistance to bone replacement and the integration with the surrounding normal non-treated cartilage and the heterogeneity and lack of standardized bioprocessing [99, 100]. The potential tumorogenesis in long follow-up was not confirmed by recent studies and the use of MSCs is probably a safe procedure [100-102].

The culture of these stem cells in a matrix and subsequent application onto a cartilage defect to treat a cartilage lesion is a new promising therapeutic approach so called a MASI procedure (matrix autologous stem-cells implantation) and can be classified as the 4th ACI generation. DeNovoR ET (Zimmer, Warshaw, Indiana) is a chondroconductive off-the-shelf matrix seeded with allogenic fetal chondrocytes is already available and clinical trials are underway. CARTSISTEM is a hyaluronate based gel seeded with mesenchymal stem cells from umbilical cord blood for a one step cartilage repair procedure and clinical trials are also underway [93].

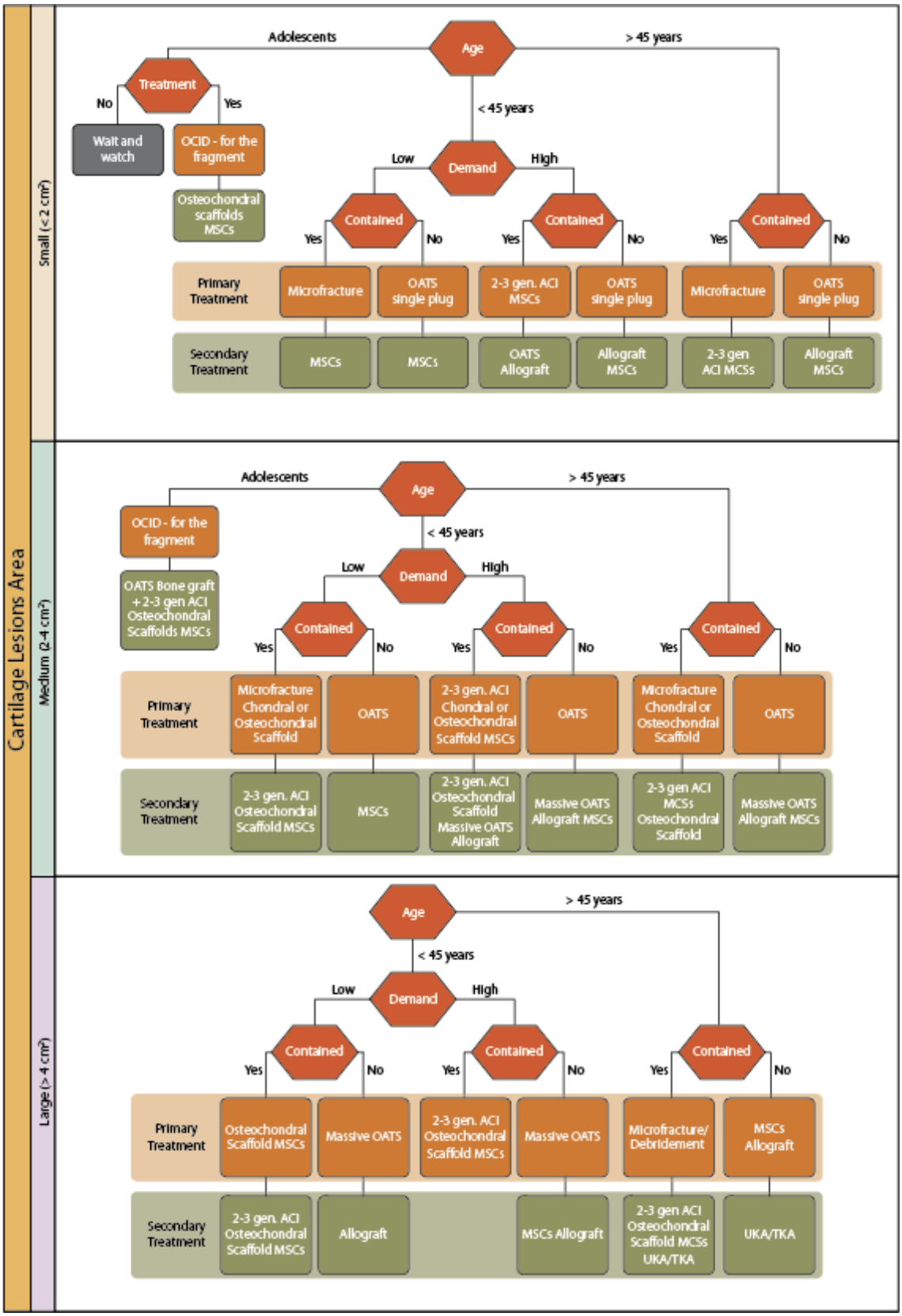


Figure 9 - *Algorithm according to the International Cartilage Repair society recommendations*

Commonly, the defect location, the size, type of the lesion and activity of the patient are the most important factors for the treatment choice. The diagram in (figure 9) is an algorithm for the decision

making in chondral and osteochondral knee injuries. In table 1 we can find some of the commercial available repair systems.

Signaling molecules including transforming growth factor (TGF-β), insulin-like growth factor (IGF), bone morphogenetic proteins (BMPs), and to a lesser extent fibroblast growth factors (FGFs) and epidermal growth factor (EGF), can improve chondrogenesis in *in vitro* studies when used isolated or associated. Important developments are expected in this field [97].

Table I – *commercial available cartilage repair systems*

PRODUCT NAME	MAIN MATERIAL	*TRIALS*
ACI PROCEDURES		
ChondroCelect® TiGenix, Leuven , Belgium	10,000 cells/microlitre suspension (Dulbecco`s Modified Eagles Medium)	*First approved cell-based product in Europe*
Carticel® Genzyme Biosurgery, Cambridge, MA	12 million cells suspension	*First FDA-approved cell therapy product*
Chondro-Gide® GeistlichBiomaterials,Wolhusen, Switzerland	Colagen	*Improved clinical outcome associated to MF or as an ACI procedure*
MACI® Genzyme Biosurgery, Cambridge, MA	Porcine type I/III collagen	*Phase III trials* *Improved outcome in case series in comparison with OAT and MF*
CaReS- ® Ars Arthro, Esslingen, Germany	Rat-tail type I collagen	*Improved clinical outcomes in a multicenter study with 116 patients/ follow up: 30 months*
NeoCart ® Histiogenics Corporation, Waltham, MA	Bovine-type I collagen Chondrocyte culture in a bioreactor	*Phase III trials*
Hyalograft C ® Fidia Advanced Biopolymers, Abano Terme, Italy	HYAFF 11 - esterified derivative of hyaluronate	*Improved clinical results even when compared with MF* *Improved clinical outcome in case series reported in 62 patients/ follow up: 7 years*
Cartipatch ® Tissue Bank of France	Agarose-alginate	*Phase III trials* *Improved clinical outcome in case series reported in 17patients / follow up: 24 months*
Bioseed C® BioTissue Technologies, GmbH, Freiburg, Germany	Copolymer of PGA, PLA and PDS - fibrin glue	*Phase III trial* *Improved clinical outcomes in in case series reported in 52 patient/ follow up: 4 years*
BioCart II ProChon BioTech Ltd., Ness Ziona, Israel	fibrinogen + hyaluronan	*Phase II trial* *Improved clinical results incase series reported in 31 patients / follow up: 17 months*
DeNovo ET® Zimmer, Warshaw, Indiana	Matrix + allogenic fetal chondrocytes	*Phase III trial*
Cartsystem	Sodium hyaluronate + allogeneic umbilical cord MSCs	*Phase II trial*
GRAFT		
DeNovo NT ® Zimmer, Warshaw, Indiana	Matrix + allogenic chondrocytes	*Good clinical outcomes in few studies reported*
CAIS® Depuy-Mitek, Raynham MA	Glue + autologous morcelleied cartilage	*Phase III trial*
CELL FREE SCAFFOLD		
TruFit® Smith & Nephew,Andover,MA	PLGA-calcium-sulfate biopolymer bilayer porous	*Suspended commercialization*
BST-CarGel® Biosyntech, Quebec, Canada	chitosan + glycerol phosphate	*Phase III trial* *Better outcomes than MF treatment in a 5 years follow-up*
CaReS-1S® Arthro- Kinetics, Esslingen, German	Rat-tail type I collagen	*Animal trials* *Short case series in adults*
MaioRegen® Fin-Ceramica S.p.A., Faenza, Italy	hydroxyapatite-collagen 3D tri-layers	*Few studies*

In conclusion, as we wait for new improved treatment techniques to achieve hyaline cartilage in the repaired tissue, more rigorous prospective, adequately powered and randomized clinical trials with the available treatments are needed to find the best cost-effective treatment for our patients. Despite the extensive efforts to develop an effective solution over the last century, there is still a paucity of clinical options of treatment for the cartilage lesions. We hope and believe that recent developments in the field of tissue engineering with all those materials, cellular approaches and repair cartilage enhancer's will find new solutions useful for cartilage lesion treatments.

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CHAPTER III

Clinical Trials and Management of Osteochondral Lesions

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ABSTRACT

Osteochondral lesions are a frequent and important cause of pain and disability. These lesions are induced by traumatic injuries or by diseases that affect both the cartilage surface and the subchondral bone. Due to the limited cartilage ability to regenerate and self-repair, these lesions tend to gradually worsen and progress towards osteoarthritis. The clinical, social and economic impact of the osteochondral lesions is impressive and although therapeutic alternatives are under discussion, a consensus is not yet to been achieved. Over the previous decade, new strategies based in innovative tissue engineering approaches have been developed with promising results. However, in order for those products reach the market and help the actual patient in an effective manner, there is still a lot of work to be done. The current state of the implications, clinical aspects and available treatments for this pathology, as well as the ongoing preclinical and clinical trials are presented in this chapter.

Key words

Musculoskeletal injuries

Bone repair

Cartilage repair

Tissue engineering

Osteochondral

Clinical studies

INTRODUCTION

Articular cartilage (AC) originated from the mesenchymal, more precisely from the skeletal blastemal, is a unique and highly specialized connective tissue, with 2 to 4 mm thickness, that covers the surface of diarthrodial joints [1]. Forming a very smooth, bright and sliding surface that facilitates pain-free movements during skeletal motion, this hyaline tissue has special mechanical properties. Composed primarily of water (65-80%of the wet weight), cells and macromolecules, AC possesses the unique ability to absorb shock impacts, support heavy and repetitive loads, and withstand wear and tear over the course of a lifetime. Water is mainly dispersed in the interfibrillar space of the matrix, thus assuring the diffusion of the nutrients and creating a moving load-dependent phase that provides flexibility, deformability and resilient strength [1]. Chondrocytes (1-5% of total tissue volume) are the sole cells found in the lacunae of the cartilage and are responsible for the synthesis and repair of the extracellular matrix (ECM) environment [2, 3]. The ECM is primarily composed of collagens (10-20%), glycosaminoglycans(GAGs) and proteoglycans (PGs) [3]. There are others constituents that can be found in the matrix, namely non-collagen proteins, glycoproteins, monosaccharides and oligosaccharides [4].

Collagen type II represents the most abundant (90-95%) form of collagen in the AC. Collagen type X, XI and, although in minor quantity, collagen type V, VI and IX are also present in the AC [4]. Collagen is a reinforcing structure of the water-proteoglycans gel phase of the matrix that increases the tensile strength and facilitates the anchorage of others macromolecules and the mineralization process. Depending on the mechanical demands, the collagen orientation varies through the thickness of the AC. The specific GAGs of physiological significance in the AC are hyaluronic acid (also known as hyaluronan), chondroitin sulfate, keratan sulfate and dermatan sulfate, all forming unbranched chains of repeating disaccharides. The PGs, which represent the second-largest group of macromolecules in the ECM of the AC, are macromolecules produced by the chondrocytes that consist of a "core protein" with one or more covalently attached glycosaminoglycan (GAG) chain(s). The AC contains a diversity of PGs that are essential for normal function, namely decorin, biglycan, fibromodulin and aggrecan. The largest in size and most abundant by weight is aggrecan, a proteoglycan that possesses over 100 chondroitin sulfate and keratan sulfate chains. Aggrecan is able to interact with hyaluronan chains and fill the interfibrillar space of the AC matrix and constitutes approximately 90% of the total cartilage matrix [4]. Four different zones or layers could be easily identified in a histological examination of AC: (1) the superficial zone(10-20% of articular

cartilage thickness), which contains collagen fibers packed tightly and aligned parallel to the articular surface, and a high water content and low PG concentration; (2) the middle or transitional zone (40-60% of AC thickness) with spherical and probably metabolic active chondrocytes showing collagen fibers in a less organized pattern; (3) the deep zone (30% of AC thickness), which contains ellipsoid cells that line up in a columnar fashion perpendicular to the joint surface and presents the highest PG concentration and the lowest water content; and (4) the calcified zone (5% of AC thickness), which plays an integral role in securing the cartilage to bone, by anchoring the collagen fibrils of the deep zone to the subchondral bone, and blocks the blood, neural or nutrients passage. Therefore, the nutrition to the articular cartilage is provided by the synovial and articular fluid in a very low oxygen tension environment [1, 3, 4]. Due to the absence of vasculature and nerve supply, cartilage has a low regenerative capacity. Thus, once injured, cartilage is much more difficult to repair.

Underneath the cartilage is the subchondral bone. Together, the AC and the subchondral bone form the osteochondral unit, which is a functional unit uniquely adapted to assure the transfer of loads across the diarthrodial joint. The subchondral bone plate constitutes the more superficial layer of compact cortical bone of the subchondral bone. Under the subchondral bone plate, a transitional subchondral spongiosa zone completes the osteochondral unit [5].Supplementary to its role as a mechanical shock absorber, the subchondral bone possesses another important metabolic function. It is richly perforated by hollow spaces that allow the invasion of arterial and venous vessels, as well as of nerves, up to the calcified cartilage from the spongiosa. Therefore, the subchondral bone also nourishes the deeper cartilage layers, providing more than 50% of its glucose, oxygen and water requirements [6].

Chondral and osteochondral lesions are frequently observed by arthroscopy and can be diagnosed in 60%-66% of all patients submitted to an arthroscopic procedure [7-11]. A retrospective analysis of 25,124 knee arthroscopies found chondral lesion in 60% of the patients [7]. Of these chondral lesions,67% were classified as localized focal osteochondral or chondral lesions [7], 29% as osteoarthritis (OA), and, in 2% of the cases, a osteochondritis dissecans was diagnosed [7]. The medial condyle (34-58%) and the patella (11-36%) are the most frequent localizations of cartilage lesions in the knee [7, 8]. The most common cartilage lesion is a traumatic single lesion affecting a patient over 50 years old with a mean area of 2.1 cm^2 [8]. In the eighteenth century, Hunter observed, when talking about articular cartilage, that “once destroyed, is not repaired” and that the evolution to degenerative changes and OA is to be expected [12-15]. OA affects approximately

15% of the population and this number will double by the year 2020 due to population ageing and obesity [15]. The lower extremity is more commonly affected and researchers estimated the lifetime risk of developing symptomatic knee OA to be about 40% in men and 47% in women [15]. The direct and indirect costs of OA have been appraised at $65 billion per year [16]. Although a common location of osteochondral defects is the knee, any other joint could be affected by a osteochondral defect [17].

Several therapeutic alternatives are available for the treatment of chondral and osteochondral lesions, but a definitive and consensual treatment for the cartilage regeneration has not yet been found. In order to repair an osteochondral defect, the needs of the bone, cartilage and the bone-cartilage interface must be taken into account [17]. A better understanding and knowledge of the cartilage and bone structure, of the biological and mechanical properties, and of the bone-cartilage interface will improve the therapeutic alternatives. Tissue engineering has been proposing single, biphasic and multiphasic scaffolds, cell therapies and bioactive molecules as advance therapies for cartilage regeneration and repair [17].

Clinical Management of Osteochondral Lesions

A traumatic chondral or osteochondral lesion usually involves the repeated application of a torsional and shear force or of a high energy force [18]. Advanced osteoarthritis and diseases involving necrosis of the subchondral bone due to different causes, including ischemia or repetitive trauma, can also be responsible for the development of osteochondral lesions [19]. Osteochondral lesions affect mostly the knee, particularly the medial femoral condyle (69%) (figure - 1), the weight-bearing portion of the lateral femoral condyle (15%), the inferomedial pole of the patella (5%) and the trochlear fossa (1%)[19]. The other most commonly affected sites are the talar dome, the elbow, the shoulder, the wrist and the hip [19]. The diagnosis is difficult. The symptoms are unspecific, and the radiologic examination is negative in an early stage of the disease. Only a magnetic resonance imaging (MRI) is helpful as a diagnosis procedure for a cartilage lesion in an initial stage. The most important complaint is pain. It can be described in many ways and may have a mechanical rhythm that worsens gradually after the start of a new activity, especially when a previous trauma is involved [9]. Pain can be exacerbated for numerous reasons: from walking to more intense sport activities [9]. Associated symptoms such as locking, pseudo-locking and giving-way can be reported when loose-bodies or associated lesions as meniscal tears or ligamentous injuries are present [20]. A methodic and exhaustive physical examination of the affected joint and

of the contra-lateral joint is mandatory. Joint swelling or joint effusion, crepitation, lameness, limitations of the range of motion of the affected joint are often present [21].

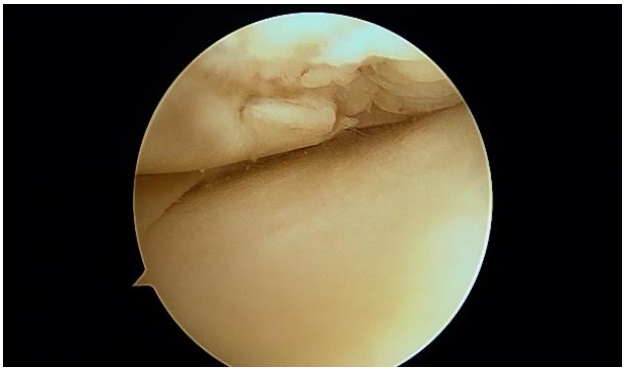

Figure 1 - *Chondral lesion*

Although radiographic studies are not very helpful in diagnosing cartilage lesions in an early stage, they can be useful for detecting osteochondral lesions, osteoarthritis, osteochondritis dissecans, loose-bodies and limb malalignment [21]. In special views, such as a 45° posteroanterior weight-bearing view of the knee (none as a “tunnel" view), a narrowing of the joint space of more than 2mm when compared with the contra-lateral knee can diagnose a major cartilage lesion of the affected knee [22, 23]. MRI confirms the clinical diagnosis and is a valuable method not only for the characterization of the lesion, but also for the evaluation of the stability and viability of the osteochondral fragments. Moreover, it is useful for staging the disease, for the follow-up evaluation, for monitoring the effects of chondral pharmacologic and surgical therapies, and in cartilage scientific research, namely for the quantitative or semi-quantitative assessment of cartilage [19, 24, 25]. MRI is helpful in the differential diagnosis of osteochondral fractures and stress fractures, which must be differentiated from osteochondral lesions [19], and to understand all the associated lesions, such as subchondral cysts and meniscal or ligamentous injuries [21]. MRI is a noninvasive procedure able to identify a cartilage lesion in a very early stage due to its ability to detect metabolic water content and structural defects. Therefore, this technique can diagnose cartilage lesions before arthroscopic observation [24, 25]. Despite the relevance of the information provided by MRI, arthroscopy remains the chosen technique for diagnosis and validation procedure of new therapeutic approaches [24].

For the correct evaluation of the severity of the cartilage lesion and its clinical implications in the treatment and outcomes, several classification scores were developed [26]. The popular Outerbridge classification score intended to classify the cartilage lesions according to macroscopic aspects, depth and extension of the cartilage injury [27]. Grade 1 included cartilage lesions presenting softening and swelling. Lesions with fissuring or fragmentation of the cartilage surface that do not exceed 1.5 cm in diameter were classified as Outerbridge grade 2. In grade 3, fissures

and cracks of the cartilage surface are present in an area of more than 1.5 cm. Grade 4 of the Outerbridge classification score included the lesions where the cartilage is eroded down to the bone [27]. Despite the simplicity and the dissemination of the Outerbridge classification score, some limitations are present[28]. Outerbridge classification score is mainly focused on the depth of the lesion with very little attention to its size. For example, a narrow deep cartilage lesion is classified as a grade 4, according to the Outerbridge classification score, but a lesion with a potentially worse prognosis, like an extensive partial thickness, is also classified as a grade 4 in this grading system (figure -2). Due to the limitations of the Outerbridge classification score aforementioned, the International Cartilage Repair Society (ICRS) developed a five grade cartilage lesion classification system based on the macroscopic evaluation and the depth of the cartilage defect [29]. In grade 0, the cartilage is normal. Grade 1A includes superficial cartilage lesions with a cartilage softening and/or superficial fissures. Grade 1B includes superficial lesions where fissures and cracks are present. In grade 2, cartilage lesions present a defect extending down to less than 50% of the cartilage thickness and fraying is present. In grade 3, the cartilage defect has to extend down to more than 50% of the cartilage thickness as well as down to the calcified layer. When there is complete loss of cartilage thickness and the underlying bone is exposed, the cartilage lesion will be classified as a grade 4 (figure-3).

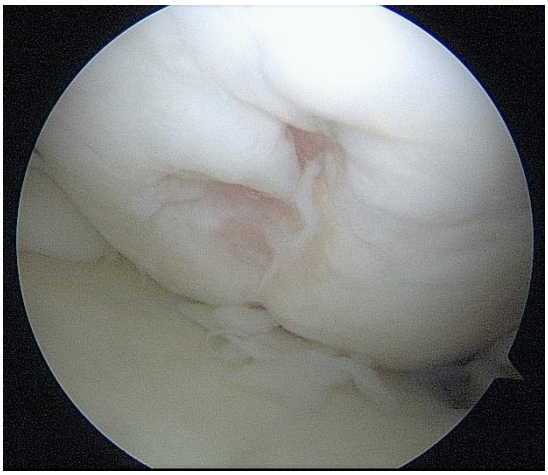

Figure 2 - *Grade IV chondral lesion*

Osteochondritis Dissecans (OCD) is a disorder characterized by the degeneration or aseptic necrosis of the subchondral bone followed by fragmentation of the overlying cartilage and that can progress to osteoarthritis [19, 30]. As repetitive trauma is thought to be the most frequent cause, the term osteochondral lesion seemed to be more appropriate than the term “osteochondritis [19, 30]. Other possible causes for this disorder are epiphyseal ossification abnormalities, endocrine imbalances, ischemia or genetic

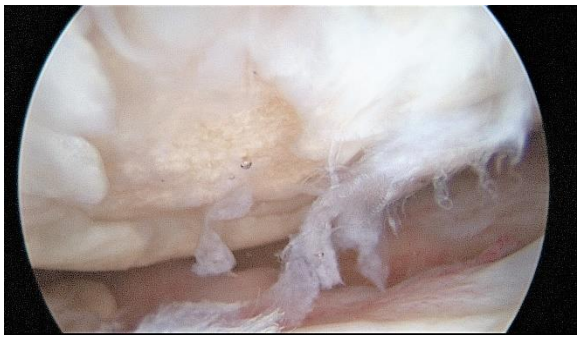

Figure 3- *Chondral lesion with the underlying bone exposed*

predisposition [19, 30]. The ICRS developed an OCD evaluation score system: in grade 0, the lesion is stable and the overlying cartilage is normal and intact; grade I includes stable lesions with some softening of the cartilage surface; grade II refers to lesions with partial discontinuity of the cartilage surface; the lesion is classified as grade III when the defect is unstable due to a complete discontinuity of the osteochondral defect; a grade IV lesion is an empty defect or a defect with loose fragments [31]. According to this grading system, grade III and IV lesions are unstable and, therefore, may have indication for surgical orthopedic treatment [19, 30].

Cartilage treatment seeks to: (i) restore a smooth articular cartilage surface and match the biomechanical/biochemical properties of normal hyaline cartilage;(ii) relieve patients' symptoms, namely pain, swelling and limping;(iii) prevent or slow the progress to osteoarthritis; (iv) lessen the morbidity of the disease and of the treatment techniques; (v) reduce the direct and indirect costs of the disease and expenses of the treatments [21].

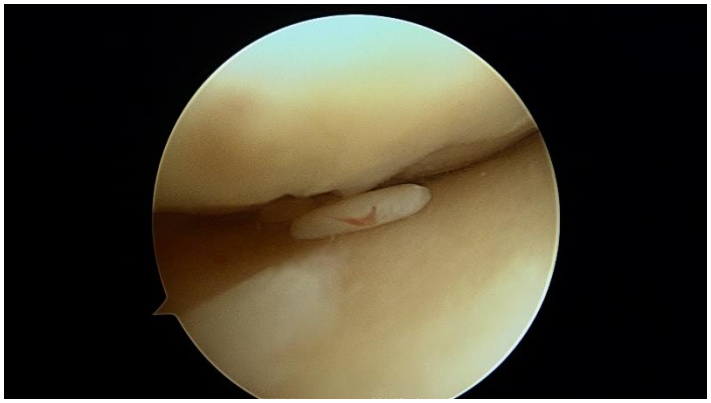

Figure 4 – *Chondral lesion with a loose body*

Palliative treatments, such as articular lavage or articular debridement, are therapeutic approaches for the relief of the patient's symptoms. An example that could clinically justify the use of a palliative treatment is a case of a symptomatic patient with pain and blocking that needs the removal of a degenerated meniscus, a loose body (figure - 4), a chondral fragment or a redundant synovia Those procedures are in decline and their effectiveness has not been proven [32, 33].

Reparative procedures, such as arthroscopic abrasion arthroplasty, abrasion chondroplasty, Pridie drilling, microfracture (MF) or spongialization, are bone marrow stimulating techniques that seek a spontaneous and natural cartilage healing by perforating through the subchondral bone to promote bleeding and consequent recruitment of bone marrow cells [34, 35]. The MF technique was initially described by Stedman. It consists in the removal of all the instable cartilage using a small awl, after which holes of 3-4mm depth separated by 3-4mm are done by perforating the subchondral bone [36]. The fibrocartilaginous repair tissue formed showed weaker mechanical properties when compared to the natural hyaline cartilage [34, 35]. MF became a very popular cartilage restoration procedure, but although good short-term clinical outcomes were reported, in the long run, or when

compared with other techniques, the impact on cartilage repair remained controversial [35, 37]. Future improvements in MF results could be achieved by administering growth factors (GF), platelet-rich plasma (PRP) or genetic engineering products [38, 39], and by using collagen membranes to cover the area treated with MF (AMIC- autologous matrix-induced chondrogenesis or BMAC - bone marrow aspirate concentrate) [37, 40, 41]. Another cartilage repair system that can be used with the MF procedure is the ChonDux™ (Cartilix, USA). This system consists of a biological adhesive and a photopolymerized hydrogel that are used combined with microfracture to enhance the stem cells migration from the bone marrow to the cartilage lesion [42].

The osteochondral autograft transplantation (OAT) is a demanding surgical procedure that consists in harvesting an osteochondral graft from the same joint or from another joint to repair the cartilage defect [34, 43, 44]. Regarding knee lesions, an osteochondral graft is harvested from a non-weight bearing area, the medial and lateral border of the condyles, the intercondylar notch or the sulcus terminalis of the lateral femoral condyle being the preferred areas [34, 43]. Mosaicplasty (MP) is a similar repair cartilage procedure, but it uses more than one graft [35, 45]. The graft presents good bone-to-bone healing interface by contrast with cartilage-to-cartilage interface [35]. Others limitations regarding these procedures concern the morbidity of the donor site, the quantity of graft that can be harvested and the congruity of the repaired articular surface [35]. The OAT technique presents its best results in small lesions (between 2.5 and 4 cm^2) located on the condyle in the weight bearing area of a young patient [11, 43]. This technique could be an alternative to MF and other regenerative cartilage failed procedures [43]. The Cartilage Autograft Implantation System - CAIS® (DePuy/Mitek, USA) is a cartilage repair procedure that uses a minced autograft cartilage dispersed in a three-dimensional scaffold based on an absorbable copolymer foam (35% polycaprolactone and 65% polyglycolic acid reinforced with a polydioxanone mesh) [46, 47].

Osteochondral allograft (OAG) transplantation could be indicated (1) for the treatment of an active patient with a massive lesion (larger than 2.5 cm^2)[11, 48], (2) for the treatment of an older patient when the alternative procedure is the arthroplasty; (3) in patients previously submitted to other cartilage repair techniques; (4) in osteonecrosis or osteochondritis dissecans cases, or (v) for the reconstructive repair of an extensive traumatic osteochondral lesion[48]. However, the reported outcomes are confusing [10, 49, 50]. This procedure presents serious limitations: (1) difficulty to obtain, preserve and manage the allografts, (2) risk of potential disease transmission, (3) immunogenicity of the allograft, and (4) surgical difficulties in fixating the allograft and achieving a good congruity with the joint cartilage surface. Chondrofix® allograft (Zimmer, USA) is considered

the first off-the-shelf osteochondral allograft. It combines donated human cancellous bone and decellularized hyaline cartilage and is recommended to repair grade III and IV osteochondral lesions in a single, less-invasive procedure[51]. DeNovo NT (Zimmer, USA) is another allograft cartilage repair procedure that consists in securing allogeneic fetal chondrocytes into the cartilage defect with fibrin glue [46] .

When the lesion has a viable part and the detached fragment presents no serious damage signs, the fixation in situ of the fragment could be the most appropriate therapeutic alternative, particularly in a young and/or active patient. After debridement of the lesion site and removal of blood clots and fibrous tissue, the fragment is then fixated in place. Several fixation devices are available: Kirshnerwires, compression screws, Herbert screws, osteochondral plugs, biodegradable pins and screws with acceptable clinical results [52, 53].

More than 20 years ago, Peterson and Brittberg, gave way to a new era in cartilage repair [54]. Their innovative two-step regenerative procedure consisted in collecting approximately 200-300 mg of a patient's articular cartilage in order to harvest chondrocytes that were then cultured and expanded. During the second stage of the process, the expanded chondrocytes were implanted into the defect and subsequently covered with a periosteal flap. The periosteal patch must be sutured water-tight to the surrounding cartilage to contain the injected suspension [11, 18, 43]. This technique is named as the autologous chondrocyte implantation (ACI) procedure. Since Brittberg et al. first described the ACI procedure, in 1994, as a treatment for chondral knee lesions [54], modifications have been introduced, thus resulting in an evolution from a first-generation to a second-generation and third-generation ACI. This technique became one of the most important surgical alternatives in the treatment of chondral lesions of the knee and its use has extended to the treatment of chondral lesions in other joints, such as the ankle, shoulder, hip and wrist [18, 21, 55]. ACI is recommended for younger patients who present symptoms of joint pain and swelling related to a chondral articular lesion. The first-generation ACI procedure was associated with a series of complications, such as periosteal graft hypertrophy, periosteal delamination, immune reaction, technical difficulties in fixating the periosteal flap, large surgical exposition, time consuming and joint stiffness [18, 55]. Carticel® (Genzyme Biosurgery, USA), an autologous cellular product commercialized since 1995 [56], is one of the techniques included in the first-generation ACI, defined as a two-stage procedure with implantation of cultured chondrocytes under a periosteal ACI (PACI).

In second-generation ACI, a membrane of porcine type I/III collagen was used (ACI-C) to cover the treated lesion. This technique claimed a decrease operating time and reduced graft complications [18, 55]. One system used in both first- and second-generation ACI is ChondroCelect® (TiGenix, Belgium), a unique cell-based cartilage repair technique that involves the combination of cultured cells with a biodegradable type I-III collagen patch. In this technique, a gene expression score was used during the isolation and expansion of chondrocytes to identify and predict the cells ability to form hyaline cartilage. ChondroCelect® was the first cartilage repair system commercialized in Europe, but it was withdrawn from the market in 2016 [47]. Chondro-Gide® (Geistlich Pharma, Switzerland), also used in first and second-generation ACI, is a porcine collagen type I/III bilayer matrix that promotes support and adhesion of autologous cultured chondrocytes in cartilage repair [41, 57].

The third-generation ACI, a matrix seeded with autologous chondrocytes is used for cartilage repair [12]. This matrix, commonly referred to as matrix-assisted chondrocyte implantation or MACI (a trademark of Genzyme), has a specific and adapted mechanical profile with chondroinductive or chondroconductive properties that allows for a more homogenous distribution of the chondrocytes and that does not require a fixation procedure [18].

There are different types of scaffolds currently used in clinical settings [58]:

1. BioSeed®-C (Biotissue Technologies, Germany) is a fibrin and polymer-based scaffold composed of polyglycolic/polylactic acid and polydioxanone[59];
2. Cartipatch® (Tissue Bank of France, France) is a monolayer agarose-alginate hydrogel scaffold[60, 61];
3. Hyalograft®-C (Fidia Advanced Biopolymers, Italy) is a hyaluronic acid-based scaffold[62];
4. MACI®(Genzyme, USA) uses a purified porcine collagen matrix to build the matrix scaffold[58];
5. Novocart®3D (TETEC Tissue Engineering Technologies AG, Germany) utilizes a collagen-chondroitin-sulfate based membrane[63];
6. NeoCart® (Histogenics, USA) implant is produced using a patient's own chondrocytes, which are then expanded and embedded in a type I collagen scaffold and incubated under low oxygen tension and variable mechanical pressure[64];
7. BioCart II™ (ProchonBioteK, Israel) is a matrix-assisted autologous chondrocytes implant made of human fibrin and hyaluronic acid that contains a apatient's own

cartilage cells after being expanded in a medium supplemented with a specific fibroblast growth factor[65];

8. CaRes® – Cartilage Regeneration System (Arthro-Kinetics, Germany) is a collagen type I matrix colonized with autologous cartilage cells for cartilage repair[66].

The fourth-generation of ACI procedures, also known as a MASI procedures (matrix-induced autologous stem-cells implantation), a matrix seeded with stem cells is used to treat the cartilage lesion. DeNovoET® (Zimmer, USA) and CARTYSTEM® (Medipost, Korea) are matrices seeded with allogeneic cells (juvenile allograft chondrocytes and allogeneic umbilical cord blood-derived mesenchymal stem cells, respectively) that are already available and with ongoing clinical trials[67, 68]. Although ACI procedures are linked to better clinical outcomes, especially when compared to other techniques, such as MF or MP [18, 43, 67, 69-71], these clinical improvements are not always statistically significant [11, 18, 55, 67, 69, 71-73].

Instead of cellular-based strategies, acellular scaffolds are becoming a feasible alternative for repair of cartilage defects: TruFit® (Smith & Nephew, USA), Maioregen®(FinCeramic, Italy), BST-CarGEL® (Smith & Nephew,England), CaRes-1S® (Arthro-Kinetics, Austria), CRD-Cartilage Repair Device (Kensey Nash Corp, USA), ChondroMimetic™ (TiGenix, Belgium), Vericart™ (Histogenics, USA), and Agili-C™(CartiHeal, Israel).

TruFit® is a bilayer porous poly(lactic-co-glycolic) acid–calcium sulfate biopolymer scaffold used in the treatment of OC defects. Its effectiveness, however, was not proven [74, 75] and the product is no longer available. Maioregen® is a scaffold with a porous three-dimensional tri-layer composite structure: the upper layer, consisting of Type I collagen, reproduces the cartilage surface; the intermediate layer is composed of magnesium-enriched hydroxyapatite and collagen and simulates the tide-mark; and the lower layer consists of magnesium-enriched hydroxyapatite, mimicking the subchondral bone [76]. BST-CarGEL®, composed of a mixture of chitosan and glycerol phosphate, was developed as a soluble polymer scaffold to stabilize the blood clot in the cartilage defect [77, 78]. CaRes-1S® is a collagen type I matrix cell-free/"cell-catcher" scaffold developed for extensive cartilage and osteochondral lesions [79, 80]. Cartilage Repair Device (Kensey Nash Corp.) is a biphasic scaffold intended to be implanted at the site of focal articular cartilage lesions or OC lesions. The chondral phase consists of a unique bovine collagen type I matrix. The subchondral phase consists of beta-tricalcium phosphate(β-TCP) mineral suspended within a porous bioresorbable synthetic polymer scaffold [51]. ChondroMimetic™ (TiGenix, Belgium) is a biocompatible, bilayered off-the-shelf scaffold composed of collagen, glycosaminoglycan and

calcium phosphate that is used for the arthroscopic repair of small lesions [51]. Vericart™ is an acellular double-structured collagen scaffold that seeks to attract chondrocytes and stem cells to promote cartilage repair [42]. Agili-C™ is a biphasic osteochondral implant consisting of two layers: a bone phase made of calcium carbonate in the aragonite crystalline form, and a superficial cartilage phase composed of modified aragonite and hyaluronic acid [51]. The last solution when in the presence of an advanced osteochondral lesion that affects the patient in a painful and restrictive way is a partial or total prosthetic replacement [81]. The treatment for chondral and osteochondral lesions depends on (1) the type, grade, location, thickness and size of the lesion, (2) the age and activity of the patient, (3) previous treatment, (4) associated lesions and, (v) systemic diseases [82]. Some orientations and guidelines for the treatment of osteochondral lesions were presented by several authors. In figure - 5, Vaquero et al. proposed an algorithm for

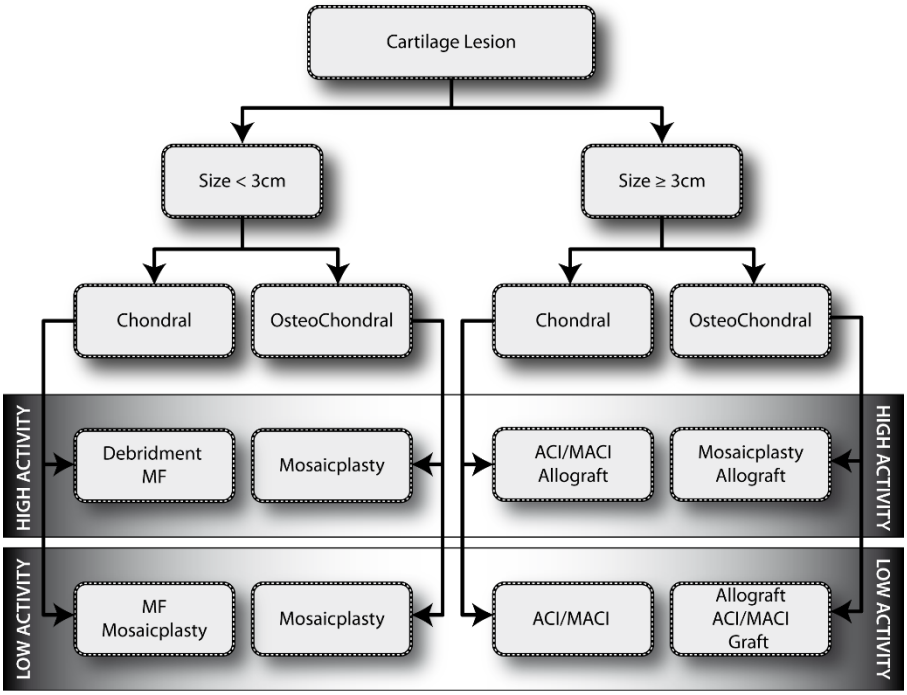

Figure 5 - *Algorithm for chondral lesions treatment*

the treatment of cartilage lesions. A consensus regarding the treatment of cartilage lesions has not yet been achieved [21] and the cost-benefit ratio of the current techniques are not entirely known [11]. The algorithm proposed by the ICRS committee for the treatment of osteochondral lesions is described in figure-6.

PRECLINICAL AND CLINICAL TRIALS

Pre-clinical and clinical trials are very complex and time-consuming steps prior to the introduction of a new therapeutic formulation in the market. During preclinical study, special attention must be

given to the choice of the animal model. For cartilage repair, these tests should be conducted in a large animal model, such as goats, horses or sheep, and should last enough time and have sufficient dimension to obtain the necessary evidences and allow for a robust analysis [83, 84]. Smaller animal models could be of interest in proof-of-concept studies, but its use presents important translational limitations : (1) the smaller volume of the cartilage defect, (2) the smaller thickness of the cartilage, and (3) the high degree of flexion in those small animals and consequent partial weight-bearing condition, are important drawbacks when compared to the human condition [26]. The biocompatibility and sterilization procedures of all the material during the process, including the manufacturing process, as well as the quality and the correct amount of the collected cells needed to be appropriate to the size of the lesion and to respect the limits of the population doubling/passage number. Tests regarding the biomechanical properties, tissue integrity and morphological characteristics of the repaired cartilage are also demanded. Biodistribution and toxicity studies could be necessary depending on the specific characteristics of the therapeutic product [83, 84].

The clinical trial should be held in hospitals, universities, doctors' offices or other locations under the direction of a principal investigator leading a multidisciplinary team and reviewed by an institutional review board [85, 86]. The research plan or protocol is designed to find answers to specific questions while assuring the health safety of the participants [85, 86]. The protocol should contain complete information about the study, namely (i) data related to the reasons for promoting the study, (ii) length and schedule of the clinical trial, (iii) number of participants involved, and (iv) drugs, techniques or treatments tested. Regarding the selection of the participants, the exclusion and inclusion criteria must be well-defined and recorded according to the protocol to assure the eligibility of the participants. The participants should be thoroughly informed and provided with answers to all possible questions in the informed consent document [85]. The trial should present a clear definition of the selected population; associated pathologies; cartilage lesion etiologies; treatments indications; type, size, localization and grade of the lesion (using a score such as the ICRS score system); and the previous failed treatments [83]. Scoring scales, such as the Knee injury and Osteoarthritis outcome score (KOOS), the IKDC subjective scale, the Lysholm score, the ICRS objective scale, an MRI with or without histological evaluation, an X-ray or an arthroscopic evaluation, are strongly recommended as confirmatory trials [83].

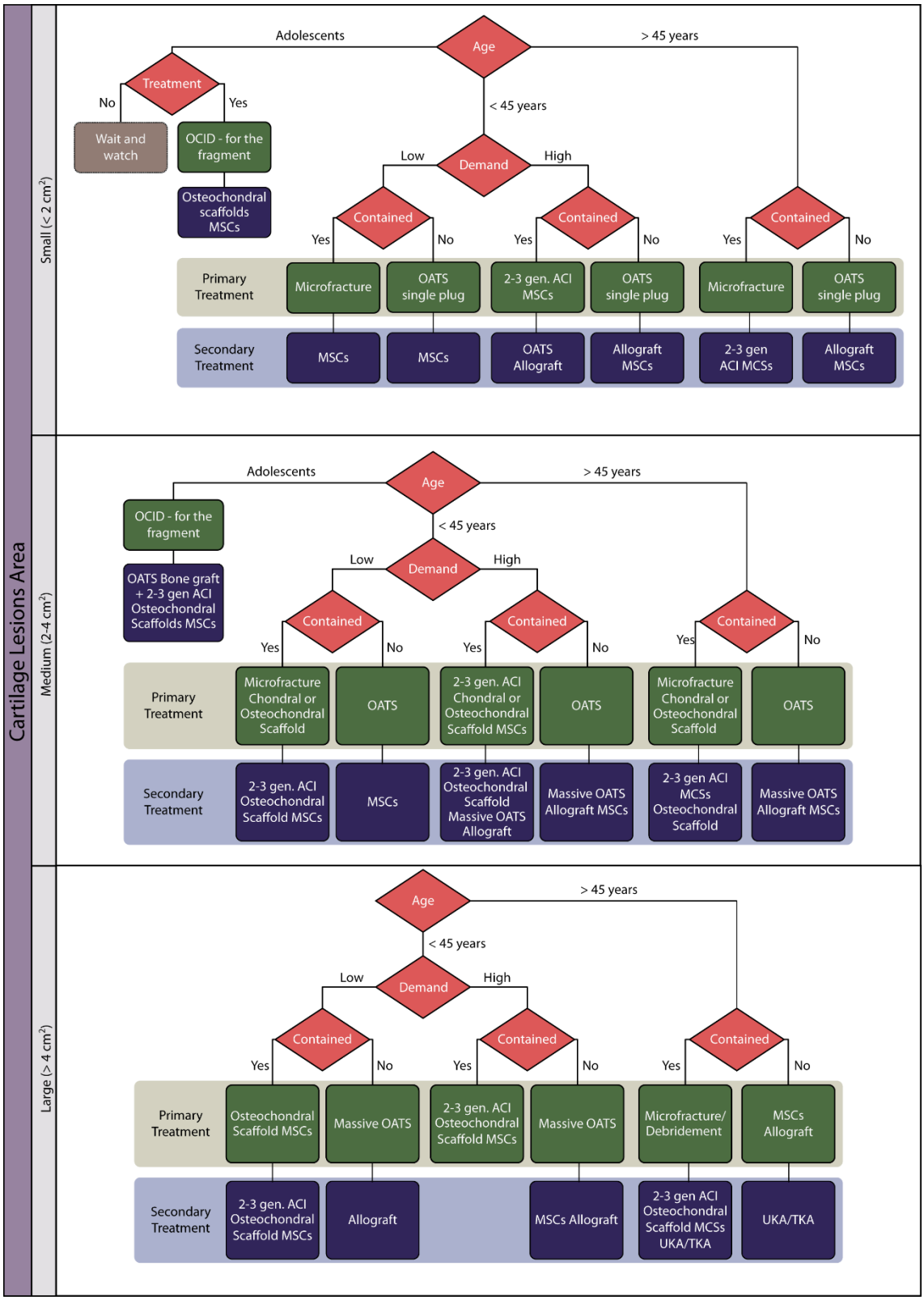

Figure 6 - *Algorithm for chondral lesions treatment according ICRS*

Special care is required concerning the design of the control group of the clinical trial. Standard therapy or the best standard of care with a centralized authorization should be used in this group. For example, microfracture remains the best option for the treatment of lesions with less than 4cm^2

[83]. Standardization of associated therapies is strongly advised: surgical protocols, symptomatic treatments, peri-surgical procedures, rehabilitation protocols and follow-up programs. A root-cause analysis is recommended for the cases where treatment failed so as to identify the cause of failure. Special attention has to be paid on long-term structural changes, such as local histological or MRI detectable changes, rates of treatment failures, as defined through relevant investigation techniques, including reoperation for revision purpose [83].

Clinical trials or interventional studies are often needed for the investigation of new approaches in the field of chondral and osteochondral repair studies. In clinical trials, the participants are submitted to specific treatments or interventions that have been previously established in the research plan. Different points have to be included in the plan, namely changes in participants' behavior and recovery protocols.[85]. Clinical trials are divided into different phases according to the stage of development of the drug or treatment. The five different phases are described in table - 1. In observational studies, researchers assess the result of interventions or procedures as part of the patient's standard medical routine treatment.[85]. In table - 2, the ongoing clinical studies are presented in accordance with the Food and Drug Administration (FDA) data based on the use of the term osteochondral [86]. Open studies could be recruiting participants or not yet recruiting. Closed studies status could be active not recruiting, completed, terminated or withdrawn [86].

Table 1 - *Five phases of clinical trials description*

Phase	Description
Phase 0	Exploratory study involving very limited human exposure to the drug, with no therapeutic or diagnostic goals (for example, screening studies, microdose studies)
Phase 1	Studies that are usually conducted with healthy volunteers and that emphasize safety. The goal is to find out what the drug's most frequent and serious adverse events are and, often, how the drug is metabolized and excreted.
Phase 2	Studies that gather preliminary data on effectiveness (whether the drug works in people who have a certain disease or condition). For example, participants receiving the drug may be compared to similar participants receiving a different treatment, usually an inactive substance, called a placebo, or a different drug. Safety continues to be evaluated, and short-term adverse events are studied.
Phase 3	Studies that gather more information about safety and effectiveness by studying different populations and different dosages and by using the drug in combination with other drugs.
Phase 4	Studies occurring after FDA has approved a drug for marketing. These including postmarket requirement and commitment studies that are required of or agreed to by the study sponsor. These studies gather additional information about a drug's safety, efficacy, or optimal use.

https://clinicaltrials.gov/ct2/help/glossary/phase

Table 2 - *Ongoing clinical trials (Searching in https: //clinical trials.gov using the term osteochondral)*

Identifier / status	**Study name / Sponsor**	**Description study**
NCT02736318 Not yet recruiting	OD-PHOENIX in Talus Osteochondral Lesion SPONSOR: TBF Genie Tissulaire	osteochondral allograft Interventional Phase 1/2
NCT01209390 Terminated	A Prospective, Post-marketing Registry on the Use of ChondroMimetic for the Repair of Osteochondral Defects SPONSOR: TiGenix n.v.	Device: Chondromimetic Observational
NCT02345564 Active, not recruiting	Clinical and Radiological Results of Osteochondral Repair Using MaioRegen in Knee and Ankle Surgery SPONSOR: Barmherzige Brüder Eisenstadt	MaioRegen Interventional
NCT01282034 Completed	Study for the Treatment of Knee Chondral and Osteochondral Lesions SPONSOR: Fin-Ceramica Faenza Spa	MaioRegen Surgery Interventional Phase 4
NCT01409447 Unknown	Repair of Articular Osteochondral Defect SPONSOR: National Taiwan University Hospital	Biphasic osteochondral composite Interventional
NCT02430558 Not Yet recruting	Second Line Treatment of Knee Osteochondral Lesion With Treated Osteochondral Graft SPONSOR: TBF Genie Tissulaire	OD-PHOENIX Interventional Phase 1/2
NCT01410136 Terminated	Chondrofix Osteochondral Allograft Prospective Study SPONSOR: Zimmer Orthobiologics, Inc.	Chondrofix Osteochondral Allograft Interventional
NCT02308358 Withdrawn	Long -Term Outcomes of Osteochondral Allografts for Osteochondral Defects of the Knee SPONSOR: University of Missouri-Columbia	Outcomes of Allograft observational

NCT02011295 Recruting	Bone Marrow Aspirate Concentrate (BMAC) Supplementation for Osteochondral Lesions SPONSOR: Duke University	microfracture + Bone Marrow Interventional Phase 4
NCT02423629 Recruting	Agili-C™ Implant Performance Evaluation in the Repair of Cartilage and Osteochondral Defects SPONSOR: Cartiheal (2009) Ltd	AgiliC™ implantation Interventional Phase 4
NCT02503228 Recruting	Clinical Assessment of the Missouri Osteochondral Allograft Preservation System - MOPS SPONSOR: James Cook, University of Missouri-Columbia	Receiving MOPS-Preserved Cartilage Observational
NCT01554878 Completed	Observational Study on the Treatment of Knee Osteochondral Lesions of Grade III-IV SPONSOR: Ettore Sansavini Health Science Foundation	knee surgery Observational
NCT03036878 Recruting	ReNu™ Marrow Stimulation Augmentation SPONSOR: NuTech Medical, Inc	ReNu Interventional
NCT01290991 Completed	A Study to Evaluate the Safety of Augment™ Bone Graft SPONSOR: William Stanish, Capital District Health Authority, Canada	Augment Bone Graft
NCT02005861 Recruting	"One-step" Bone Marrow Mononuclear Cell Transplantation in Talar Osteochondral Lesions SPONSOR: Istituto Ortopedico Rizzoli	bone marrow cells + collagen scaffold Interventional
NCT02338375 Unknown	Safety and Efficacy of Allogenic Umbilical Cord Blood-derived Mesenchymal Stem Cell Product SPONSOR:	Cartistem Interventional Phase 1
NCT02309957 Recruting	EAGLE European Post Market Study SPONSOR: Kensey Nash Corporation	BioMatrix CD Interventional

NCT01477008 Recruting	BiPhasic Cartilage Repair Implant (BiCRI) IDE Clinical Trial - Taiwan SPONSOR: Exactech Taiwan, Ltd	BiPhasic Cartilage Repair Implant Interventional Phase 3
NCT00560664 Completed	Comparison of Autologous Chondrocyte Implantation Versus Mosaicoplasty: a Randomized Trial SPONSOR: University Hospital, Brest	Mosaicoplasty Interventional Phase 3
NCT00984594 Terminated has results	Evaluation of a Composite Cancellous and Demineralized Bone Plug (CR-Plug) for Repair of Knee Osteochondral Defects SPONSOR: RTI Surgical	Composite of Cancellous and Demineralized Bone Plug (CR-Plug) Interventional Phase 3
NCT00891501 Unknown	The Use of Autologous Bone Marrow Mesenchymal Stem Cells in the Treatment of Articular Cartilage Defects SPONSOR: Cairo University	Bone marrow mesenchymal stem cell implantation Interventional Phase 2/3
NCT00821873 Completed has results	Evaluation of the CR Plug for Repair of Defects Created at the Harvest Site From an Autograft in the Knee. SPONSOR: RTI urgical	CR Plug Interventional Phase 3
NCT01183637 Terminated	Evaluation of an Acellular Osteochondral Graft for Cartilage LEsions Pilot Trial SPONSOR: Kensey Nash Corporation	Kensey Nash Corp. Cartilage Repair Device Interventional Phase 2
NCT01159899 Unknown	Transplantation of Bone Marrow Stem Cells Stimulated by Proteins Scaffold to Heal Defects Articular Cartilage of the Knee SPONSOR: Michel Assor, MD, University of Marseille	Transplantation of Bone Marrow Stem Cells Activated in Knee Arthrosis Interventional Phase1

NCT01471236 Active, not recruiting	Evaluation of the Agili-C Biphasic Implant in the Knee Joint SPONSOR: BioPoly LLC	BioPoly RS Partial Resurfacing Knee Implant Interventional Phase 4
NCT00945399 Terminated	Comparison of Microfracture Treatment and CARTIPATCH® Chondrocyte Graft Treatment in Femoral Condyle Lesions SPONSOR: TBF Genie Tissulaire	CARTIPATCH® procedure Interventional Phase 3
NCT01473199 Completed	BioPoly RS Knee Registry Study for Cartilage Defect Replacement SPONSOR: BioPoly LLC	BioPoly RS Partial Resurfacing Knee Implant Interventional
NCT01799876 Active, not recruiting	Use of Cell Therapy to Enhance Arthroscopic Knee Cartilage Surgery SPONSOR: Fondren Orthopedic Group L.L.P.	Autologous Cell /microfracture Interventional
NCT01747681 Completed	Results at 10 to 14 Years After Microfracture in the Knee SPONSOR: Bergen Orthopedic Study Group	Microfracture Observational
NCT01347892 Active, not recruiting	DeNovo NT Ankle LDC Study SPONSOR: Zimmer Orthobiologics, Inc.	DeNovo NT Natural Tissue Graft Interventional
NCT01920373 Withdrawn	Platelet-Rich Plasma vs Corticosteroid Injection as Treatment for Degenerative Pathology of the Temporomandibular Joint SPONSOR: Kaiser Permanente	corticosteroid injection / platelet rich plasma injection Interventional Phase 1
NCT02991300 Recruiting	BioPoly® RS Partial Resurfacing Patella Registry Study SPONSOR: BioPoly LLC	BioPoly RS Partial Resurfacing Patella Implant Interventional
NCT00793104 Terminated has results	Evaluation of the CR Plug (Allograft) for the Treatment of a Cartilage Injury in the Knee. SPONSOR: RTI Surgical	CR Plug Interventional Phase 3

Conclusions

Despite all the therapeutic approaches for the treatment of chondral and osteochondral lesions, a consensus regarding a definitive treatment has not yet been achieved. Allografts, autografts or substitution arthroplasties have already proven their value in cartilage repair. Emerging tissue engineering and regenerative approaches have shown promising results and could advance new solutions for osteochondral lesions treatment, but further studies and research need to be conducted. Although tissue engineering has already shown tremendous progress, a long and difficult road in the regulatory and legal path has to be travelled in order to transform new therapeutic approaches into a clinical reality.

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CHAPTER IV

Cartilage Repair Using Hydrogels: a Critical Review of *In Vivo* Experimental Designs

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ABSTRACT

This review analyzes the outcomes and technical aspects of *in vivo* studies published in the past decade using gels and hydrogels for cartilage repair. Using PubMed search engine, original research publications during the period of 2002/01/01 to 2015/04/30 identified 115 published papers. Of these, 3 studies failed to find a statistically significant improvement of treatment group as compared to control and 18 studies did not clearly identify hyaline-like cartilage formation in the treated groups. The most frequent repaired lesion was the rabbit acute full thickness trochlear defect, using a scaffold combining a gel or hydrogel and other material. One third of the scaffolds were cell-free (35%) and the majority of the studies did not use growth factors (71%). The present review may constitute a useful tool in design of future studies, as limitations of study designs are pointed and results in terms of translation to human application is discussed.

Key Words: Cartilage repair**,** Hydrogels**,** Gels, *In Vivo*, Animal, Tissue Engineering

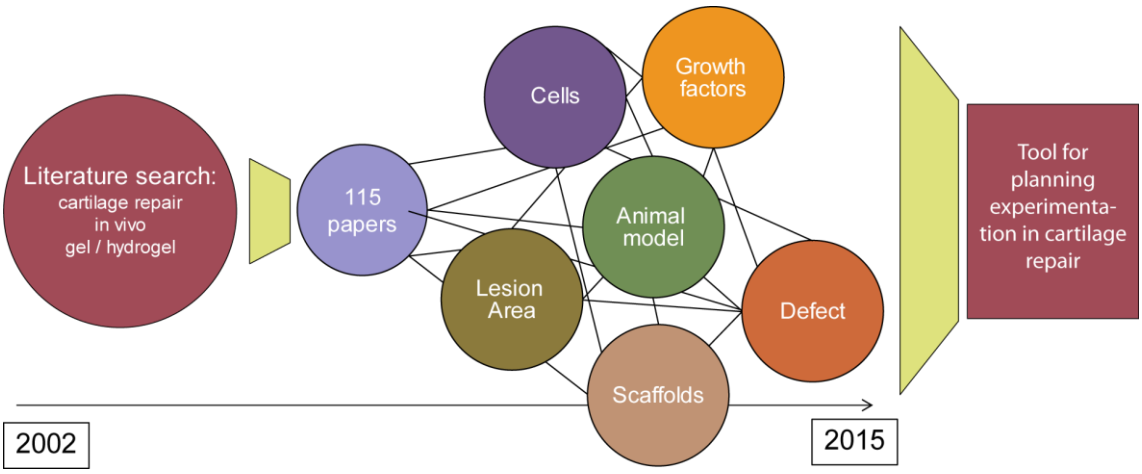


Figure 1 - Visual representation of the essence of the paper

INTRODUCTION

Articular cartilage has limited intrinsic capacity for self-repair, because of the lack of vascular, neural and lymphatic networks, as well as absence of progenitor cells [1, 2]. According to Hjelle et al., cartilage lesions were found in 60% of patients submitted to knee arthroscopy [3]. Cartilage lesions commonly progress to osteoarthritis (OA), as a final state of disease evolution [2, 4]. Presently, it is estimated that 10%-15% of adults over 60 years old show some symptoms of disease and by 2050, 130 million people will suffer from osteoarthritis, worldwide [5]. The clinical and economic impact is impressive, as the estimated direct and indirect costs related to OA has surpassed $65 billion annually [6].

At earlier stages of cartilage damage, current therapies for cartilage repair of lesions are not satisfactory as they fail to restore a normal hyaline cartilage [7-9]. Surgical approaches can include microfracture, resurfacing techniques and osteochondral grafting [8-10]. Autologous chondrocyte implantation (ACI) and matrix-assisted autologous chondrocyte implantation (MACI) are advanced approaches for regeneration of cartilage lesions [9, 10].

Microfracture and resurfacing techniques are easy to perform, cost competitive, widely adopted and well-documented techniques that relieve symptomatic patients. However, regenerated tissue is composed mostly by fibrocartilage, thus providing short-term positive results in small cartilage lesions [8, 10].

Osteochondral grafting, the direct transplantation of an osteochondral autograft (mosaicplasty) or allograft, is the only technique available that satisfactorily restores hyaline cartilage [4]. However, donor site morbidity, risk of disease transmission, possible graft-versus-host immune response (in the case of allografts) and osteoarthritic exacerbation can occur due to lack of congruency between treated and untreated surfaces, thus limiting the use of those techniques [4].

On the other hand, ACI and MACI are expensive techniques, which demand complex protocols and two different surgeries. Promising results have been reported[4], but poor consistence of clinical outcomes with time, cells and/or cartilage fragment loosening, arthrofibrosis, osteophytes development, synovitis, infection and chondromalacia have been described [4, 10].

Many of the limitations of current available treatments justify the quest for more effective approaches and development of new biomaterials for cartilage repair. Interestingly, hydrogels have attracted great deal of attention because of its performance characteristics, i.e. are soft, of synthetic or natural origin, and can form three-dimensional networks that can be tuned for its

biocompatibility, bioadhesiveness and biodegradability [11, 12]. Hydrogels present other advantageous features for tissue engineering applications [11-14], such as: extracellular matrix mimetic; swelling ability while maintaining shape; capability to undergo volume phase or sol-gel phase transitions in response to physical and/or chemical stimuli; tunable surface and bulk properties to modulate cells adhesion and thrombogenicity; support to high diffusion kinetics of nutrients and metabolic products within the construct.

There are several chemical or physical crosslinking techniques, photopolymerization, or even microfabrication technologies [13, 15, 16], which can optimize hydrogel physicochemical characteristics and biological behavior [13], and improve performance of hydrogels in cartilage tissue engineering strategies. Furthermore, hydrogels can be combined with other materials improving its properties [17-19].

The application of hydrogels as volume fillers and cell carriers can contribute significantly to the development of more effective regeneration strategies [20] in irregular shape cartilage defects. Additionally, the opportunity to treat such lesions by a single step procedure using simpler surgical protocols, in which an injectable solution is delivered by a minimally invasive procedure, can minimize significantly treatment cost, improve patient safety and comfort, and support treatment in an outpatient setting.

This review compiles *in vivo* studies reporting the use of hydrogels for repairing cartilage lesions and analyzes its performance in different animal models. A thorough analysis of experimental variables was further performed, constituting a useful tool for researchers when designing future *in vivo* studies for cartilage repair.

METHODS

Keyword-Based Search

Original research publications were identified by the use of PubMed® search engine, during the period comprised between 2002/01/01 and 2015/04/30, and using the following keywords: “cartilage”, “osteochondral”, “cartilage repair”, “tissue engineering”, “scaffold”, “cells”, “gel”, “gels”, “hydrogel” and “hydrogels”, using AND /OR Boolean operators. The terms such as “eye”, “heart”, “tooth”, “skin”, “root”, “dermal”, “dentin”, “cardiovascular”, “hepatic”, “gastric”, “gastrointestinal” and “biochemistry” were excluded.

Inclusion/Exclusion Selection

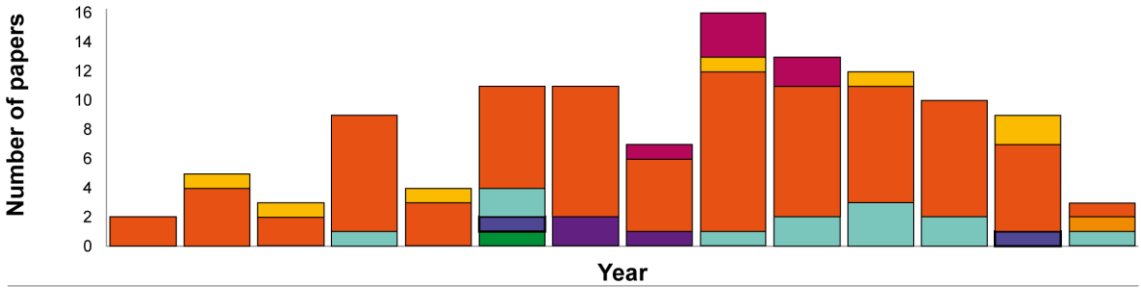
All abstracts were evaluated by four independent reviewers based on the following inclusion criteria: English language, and experimental protocol reporting *in vivo* use of hydrogels in repair of cartilage defects. The following exclusion were applied: Other language rather than English; *in vitro* studies; studies not involving use of hydrogels; studies reporting use of hydrogels in other application contexts or studies in which the hydrogel could not be considered as a scaffold. Whenever the abstract was unclear or insufficient for determination of its inclusion/exclusion, the Materials and Methods section and/or the complete publication were analyzed before a decision was made.

Evaluation and Final Selection

After selection of abstracts, a second evaluation was carried out, during which all publications were analyzed and discussed among the four reviewers in order to produce the final list of publications to be overviewed.

Full Text Review

All included articles were submitted to a full-text review. For each paper, the respective list of references was verified to identify possible relevant studies that might have been undetected through PubMed-based search.



	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
Dog						1								
Goat							2	1						
Horse						1							1	
Mini pig				1		2			1	2	3	2		1
Monkey														1
Rabbit	2	4	2	8	3	7	9	5	11	9	8	8	6	1
Rat		1	1		1				1		1		2	
Sheep								1	3	2				

Figure 2 - *Number of original scientific publications per year published between 2002 and 2014 reporting in vivo experiments on cartilage repair according to animal model.*

RESULTS

Publication Selection and Review

Keyword based search identified a total of 14295 publications. After inclusion/exclusion selection, 902 papers related to study of articular cartilage repair have been identified. Then, evaluation and final selection of those papers, according to defined inclusion and exclusion criteria, identified a total of 93 papers. During the selection process, 809 studies have been excluded due to several reasons, such as *in vitro* experimental protocol, experiments not aimed at repairing cartilage defects, or papers reporting clinical investigations. For each paper, the list of references was verified, which allowed identification of 22 additional publications. Herein, the total number of published original articles identified, reviewed and included was 115.

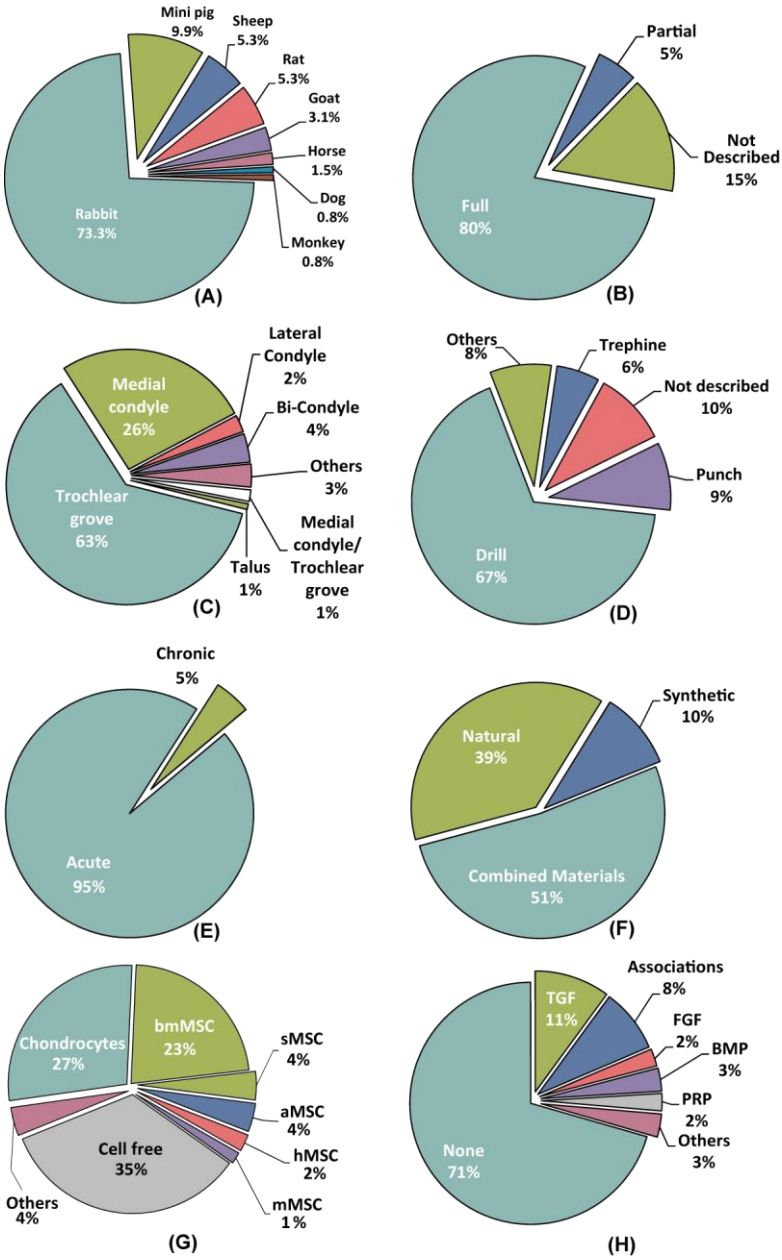


Figure 3 –
Distribution of animal models, characterization of the induced defect and lesion treatment and bioactive agents used: ***(A)*** *Animal model;* ***(B)*** *Lesion type;* ***(C)*** *Location of the lesion;* ***(D)*** *Techniques for defect induction;* ***(E)*** *Disease stage;* ***(F)*** *Type of scaffold;* ***(G)*** *Type of cells: adipose mesenchymal stem cells (aMSC), muscle mesenchymal stem cells (mMSC), synovium mesenchymal stem cells (sMSC), bone-marrow mesenchymal stem cells (bmMSC) and human mesenchymal stem cells (HMSC);* ***(H)*** *Growth Factors: Transforming growth factor (TGF), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), platelet rich plasma (PRP).*

Distribution of Publications per Year

The distribution of publications per year is shown in Figure 2. Between 2002 and 2010, the number of publications reporting *in vivo* experiments concerning cartilage repair have increased every year. After 2010, the number of publications per year appears to have stabilized between 10 and 13 papers per year.

Animal Models

Upon analysis of the publications, several outcomes were obtained regarding animal models and respective experimental protocol (Figure 3). According to Figure 3A, the rabbit model was the most common for studying cartilage repair by means of using hydrogels, comprising 73.3% of all studies. Noticeably, other animal models selected for evaluation of hydrogel performance included large animals, such as goat and sheep models, representing 3.1 and 5.3% of all studies, respectively (Figure 3A). Minipigs were the second animal more frequently used comprising 9.9% of all studies (Figure 3A).

Age and Weight of Animals

Table 1 summarizes the data regarding age and weight of animals for all the studies analyzed. The absolute ranges depend very much on the animal model. The animal model with the wider age interval was the sheep, with a relative interval of 40 to 260 weeks. As concerns weight, the animal model with the wider weight intervals were sheep and horse, with a relative interval of 22.5 to 80 kg and 307 to 439 kg, respectively.

Table 1 - *Maximum, minimum, average and mode of age and weight of animals used for in vivo experiments on cartilage repair according to animal model as reported in analyzed publications*

	Age (Weeks)				Weight (kg)			
Animal	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**
Dog[a)]	-	-	104.0	-	-	-	9.4	-
Goat	-	-	-	-	47.6	23.9	33.8	-
Horse	182	156	169.0	-	439	307	373.0	-
Mini pig	156	16	43.2	24/32/44	42.0	11.0	26.2	36.5
Monkey[a)]	-	-	312.0	-	-	-	8.0	-
Rabbit	96	8	20.8	24	4.7	1.8	3.1	2.3/3.3
Rat	12	5	10.7	12	0.4	0.3	0.3	-
Sheep	260	40	152.4	117	80.0	22.5	64.9	68.0/80.0

Abbreviations: Max - Maximum; Min - Minimum; Avg - Average

a) Only 1 study with this animal model

Number of Animals per Study

Table 2 presents the number of animals used for each study and the time interval for the time points according to each animal model. The most common number of animals used per study was 12, as this was the mode obtained for mini-pig, rabbit and sheep models. As for the duration of the studies, 12 weeks was the mode obtained for the most used animal models, rabbit and mini-pig yet ranging from 4 and 8 to 52 weeks, respectively.

Table 2 - *Maximum, minimum, average and mode of the number of animals and duration of study adopted for in vivo experiments on cartilage repair according to animal model as reported in analyzed publications* [a]

	Number of animals/Paper				Duration (Weeks)			
Animal	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**
Dog[a)]	-	-	9.0	-	-	-	10.0	-
Goat	20	4	12.5	-	24	12	19.0	24
Horse	6	6	6.0	-	32	24	28.0	-
Mini pig	27	6	14.8	12/16/18/20	52	8	20.0	1
Monkey[a)]	-	-	16.0	-	-	-	24.0	-
Rabbit	81	3	27.3	12	52	4	13.9	12
Rat	121	9	40.2	-	12	4	7.0	-
Sheep	24	3	11.4	10/12	52	3	27.0	16/52

Abbreviations: Max - Maximum; Min - Minimum; Avg - Average

a) Only 1 study with this animal model

Experimental Protocol

Type and geometry of defects

From Figure 3, it is possible to state that the most frequently induced cartilage defect was a full thickness lesion (80%, Figure 3B), done in the trochlea (63%, Figure 3C) by drilling (67%, Figure 3D) and treated at an acute stage (95%, Figure 3E).

Cartilage defect dimensions were also thoroughly analyzed, including area, depth and volume (Table 3). Most defects had a circular shape, yet 8 articles reported a rectangular or square shape[21-28]. Therefore, for comparison purposes, it was adopted the defect area to characterize surface dimension. Dimensions varied according to the animal model employed. In general, dimensions of induced cartilage defect were proportional to the size of the animal. For rat, the minimum area of the lesion was 0.6 mm^2 and for horse the maximum area was 176.7mm^2. For rabbit, the most frequently adopted animal model, the mode of the lesion area was 7.1 mm^2, Whereas the lesion area varied between 1.8 and 200 mm^2. The very large variation in defect area results from one study where the defect included the complete excision of tibial plateau [29].

Table 3 - *Maximum, minimum, average and mode of the number of lesions, lesion area, lesion depth and lesion volume adopted for in vivo experiments on cartilage repair according to animal model as reported in analyzed publications* [a]

	Number of lesions				Lesion area (mm^2)				Lesion depth (mm)				Lesion volume (mm^3)			
Animal	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**
Dog[a)]	-	-	2.0	-	-	-	19.6	-	-	-	4.5	-	-	-	88.4	-
Goat	2	2	2.0	2	28.3	19.6	24.0	-	4.0	3.0	3.3	3.0	113.1	58.9	78.9	58.9
Horse	2	2	2.0	-	176.7	176.7	176.7	-	2.8	2.8	2.8	-	486.0	486.0	486.0	-
Mini pig	8	1	2.8	2	56.7	12.6	32.3	28.3	6.0	1.0	2.6	1.0/3.0	169.6	28.3	87.1	28.3
Monkey[a)]	-	-	2.0	-	-	-	7.1	-	-	-	5.0	-	-	-	35.3	-
Rabbit	6	1	2.1	2	200.0	1.8	15.4	7.1	15.0	0.5	3.7	3.0	217.8	3.5	51.0	21.2
Rat	2	1	1.5	1	3.1	0.6	1.9	1.8	2.0	1.0	1.5	-	6.3	1.8	3.3	-
Sheep	12	1	5.0	4	50.3	8.0	32.4	28.3/38.5	12.0	2.0	6.7	12.0	423.1	32.2	205.5	77.0/339.3

Abbreviations: Max - Maximum; Min - Minimum; Avg - Average
a) Only 1 study with this animal model

Type of scaffold

From Figure 3F, it is evident that "combined materials" prevail as the most frequent type of scaffold (51%). These are composed by two or more materials of either of natural or of synthetic origin. The other types of scaffold that has been mostly investigated were natural derived scaffolds (39%) and synthetic scaffolds (10%).

When analyzing use of scaffolds with cells, about 65% of all studies analyzed involved the use of cells, in a so-called combination repair strategy. Nevertheless, about 35% of cartilage lesions where treated with hydrogels alone (Figure 3G).

Type of cells

For combination approaches where scaffolds are combined with cells, 27% of studies used chondrocytes, whereas 38% used mesenchymal stem cells (Figure 3G). A thorough description of cell types and concentrations used in the analyzed studies are displayed in Table 4. Chondrocytes were the most widely used cell type in a concentration range between 5.00×10^4 and 5.00×10^7 cells/mL, followed by mesenchymal stem cells (MSC) that have been used in a range between 1.50×10^5 and 7.20×10^5 cells/mL. We noticed that 35% of the scaffolds were cell-free.

Table 4 - *Maximum, minimum, and average number of cells used for in vivo experiments on cartilage repair according to cell type and animal model as reported in analyzed publications*

Cell Type	Cell Concentration (Cells/mL)	Animal							
		Dog[a)]	Goat	Horse	Mini pig	Monkey[a)]	Rabbit	Rat	Sheep
aMsc	Max	-	-	-	-	-	1.50E+07	1.00E+06	-
	Min	-	-	-	-	-	1.00E+06	1.00E+06	-
	Avg	-	-	-	-	-	9.00E+06	1.00E+06	-
bMsc	Max	-	5.00E+07	-	7.00E+06	1.00E+06	5.00E+07	-	7.20E+05
	Min	-	5.00E+07	-	1.00E+05	1.00E+06	1.50E+05	-	4.00E+05
	Avg	1.00E+07	5.00E+07	-	2.28E+06	1.00E+06	1.28E+07	-	5.07E+05
BNC	Max	-	-	-	1.00E+05	-	-	-	-
	Min	-	-	-	1.00E+05	-	-	-	-
	Avg	-	-	-	1.00E+05	-	-	-	-
Chondrocytes	Max	-	5.00E+06	-	5.00E+07	-	5.00E+07	5.00E+04	4.00E+07
	Min	-	1.00E+06	-	2.00E+05	-	7.50E+04	5.00E+04	1.00E+06
	Avg	-	3.00E+06	1.20E+07	1.59E+07	-	5.76E+06	5.00E+04	1.63E+07
HMsc	Max	-	-	-	-	-	1.00E+06	2.00E+07	-
	Min	-	-	-	-	-	1.00E+06	2.00E+07	-
	Avg	-	-	-	-	-	1.00E+06	2.00E+07	-
mMsc	Max	-	-	-	-	-	2.00E+06	-	-
	Min	-	-	-	-	-	1.00E+06	-	-
	Avg	-	-	-	-	-	1.50E+06	-	-
PBC	Max	-	-	-	-	-	2.36E+07	-	-
	Min	-	-	-	-	-	2.36E+07	-	-
	Avg	-	-	-	-	-	2.36E+07	-	-
Periostal cells	Max	-	-	-	-	-	-	-	8.00E+06
	Min	-	-	-	-	-	-	-	8.00E+06
	Avg	-	-	-	-	-	-	-	8.00E+06
sMsc	Max	-	-	-	-	-	1.00E+08	-	-
	Min	-	-	-	-	-	1.00E+06	-	-
	Avg	-	-	-	-	-	3.82E+07	-	-

Abbreviations: Max - Maximum; Min - Minimum; Avg - Average

a) Only 1 study with this animal model

aMSC: adipose mesenchymal stem cells; bmMSC: bone-marrow mesenchymal stem cells; bmNC: bone-marrow nucleated cells; HMSC: human mesenchymal stem cells; mMSC: muscle mesenchymal stem cells; PBC: peripheral blood mononuclear cells; sMSC: synovium mesenchymal stem cells.

Bioactive agents

Besides the use of cells with the hydrogels in combination strategies to repair cartilage lesions, growth factors have been also explored to improve quality of regenerated tissue. According to Figure 3H, published papers used at least one growth factor (29%) for repair of cartilage lesions. Transforming growth factor (TGF) was the most frequent choice, accounting for 11% of studies, whereas bone morphogenetic protein (BMP) and fibroblast growth factor (FGF) were each used in 3 and 2% of all studies respectively. Insulin growth factor (IGF), growth differentiation factor (GDF), connective tissue growth factor (CTGF) and Nel-like molecule-1 (NELL-1) account for a total of 3% of studies (Figure 3H). Noticeably, platelet-rich plasma (PRP) has also considerable expression in this context, accounting for 2% of all studies (Figure 3H).

Time points and study groups

Table 5 presents number of time points, number of study groups and number of lesions per study group for the analyzed publications. Concerning number of time points, the majority of studies included at least 2 time points, yet the number ranged from 1 time point up to 7 time points. Three study groups was the most common among all animal models, the number of lesions per study group was in average 12 -15.4, for the mini-pig and rabbit models respectively.

Table 5 - *Maximum, minimum, average and mode of the number of time points, number of study groups and number of lesions per study group adopted for in vivo experiments on cartilage repair according to animal model as reported in analyzed publications.*

	Number of time points				Number of study groups				Number of lesions/Study group		
Animal	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**	**Mode**	**Max**	**Min**	**Avg**
Dog[a)]	-	-	2.0	-	-	-	2.0	-	9	9	9.0
Goat	3	1	2.0	2	4	2	3.0	3	12	3	8.3
Horse	2	1	1.5	-	2	2	2.0	-	6	6	6.0
Mini pig	3	1	1.5	1	5	2	3.1	3	24	5	12.0
Monkey[a)]	-	-	3.0	-	-	-	2.0		-	-	16.0
Rabbit	7	1	2.3	2	9	2	3.3	3	74	3	15.4
Rat	3	1	1.8	1	7	3	3.9	3	20	13	16.8
Sheep	3	1	1.7	1/2	6	2	3.9	4	20	6	10.8

Abbreviations: Max - Maximum; Min - Minimum; Avg - Average

a) Only 1 study with this animal model

For all animal models, the number of study groups was between 2 and 9. Concerning, the number of lesions by study group (N), this index was calculated according the equation:

$$N = \frac{Nº\ of\ animals\ \times\ Nº\ of\ lesions\ by\ animal}{Nº\ of\ study\ groups}$$

According to Table 5, the number of lesions per study groups was between 3 and 74.0.

Characterization of cartilage repair

Several techniques have been used to evaluate regeneration of cartilage tissue within the induced lesions. Histological staining of cartilage explants was done in all studies, including hematoxylin and eosin staining in most of the studies, complemented with at least one of the following: alcian blue, toluidine blue, safranin O and/or Masson's trichrome staining. Immunohistochemistry staining, commonly used for identification of collagen type II and/or collagen type I, were done in 71 reports. For histological evaluation several histological scores were chosen: O'Driscoll, Pineda, ICRS, Mankin, Moran, Wakitani, Wayne, Seller´s, Caplan and Susante. In 15 papers, two of the previous scores were used simultaneously. O'Driscoll scoring was used, alone or combined with another score, in 29 publications, followed by Wakitani scoring in 20 studies and by the ICRS scoring in 19 papers. In 32 studies, no histological score was used to evaluate the quality of

cartilage regeneration. As outcome of histological evaluation, most studies have obtained statistically significant improvement in cartilage regeneration for treated groups as compared to control groups. In 3 papers [30-32], no significant histological improvement was observed between treated and untreated groups and in 4 papers [33-36] no histological differences were found between study groups. Most studies reported on the development of hyaline-like cartilage, while 8 studies [37-44] reported no cartilage like tissue or a mixture of fibrous cartilage and hyaline-like cartilage in the repaired tissue. In 14 studies [23, 30-32, 45-51], the repair tissue was not classified as hyaline-like cartilage. In 5 papers [21, 52-54], a tendency for deterioration of cartilage tissue along time was reported.

Quantitatively, gene expression was evaluated in 25 studies (21,7%). Characterization of mechanical performance of regenerated tissue was highly uncommon, as it was performed in 8 studies. Imaging evaluation including magnetic resonance imaging (MRI), micro-computed tomography (μ-CT), laser scanning arthroscopy, optical coherence tomography (OCT) was performed in 19 studies.

Side effects

Several side effects have been reported in the studies analyzed such as inflammation, degeneration, tissue hypertrophy, among others. No information was given related to this issue in 26 studies. Inflammatory response was reported in 9 studies [24, 31, 52, 55-60] including, synovitis, fibrosis and fissures. By its turn, 13 papers reported degenerative or pathological changes like osteophytes, cyst formation or bone hypertrophy. In these studies, one of the following were used: a periostal flap in a chondrocyte cell-laden scaffold [21], PRPs [61], a growth factor (TGFβ [62] [63], BMP-2 [31], FGF [52]) or cells (ASC [64], BMSC [65], MSC [25, 54], chondrocytes [66] chondrocytes/periostal cells [67]). In another study [68], the control group developed a degenerative change.

DISCUSSION

The present systematic review revealed that hydrogels used for cartilage repair include those composed by single natural or synthetic biomaterials, or by combination of these, designated as “combined materials” (Figure 3F). Advantages / disadvantages of natural and synthetic

biomaterials for cartilage repair are detailed elsewhere[69, 70]. Among the literature revised, 39% of studies proposed natural materials including collagen [21, 22, 25, 26, 28, 32, 39, 47, 50, 52, 58, 63, 64, 71-76], alginate [37, 38, 40, 48, 62, 77-80], fibrin [29, 33, 81, 82] , platelet-rich plasma [61, 83], hyaluronic acid [27, 31, 57, 84, 85], gellan gum [86], chitosan[42, 87] and sugar cane biopolymer [88]. For 10% of studies, synthetic materials included oligo(poly(ethylene glycol) fumarate) (OPF)[43, 46, 89], poly(N-isopropylacrylamide-co-acrylic acid) (poly(NiPAAm-co-AAc)) [90], poly(L-lactide-co-3-caprolactone) (PLCL) [91, 92], Si-HPMC [36], polypeptides [35, 65, 93] and α-CD-EG 4400 [94]. The scaffolds using combined materials were composed by two or more natural materials, representing 51% of the studies [17-19, 49, 60, 66-68, 95-100], by association of two or more synthetic polymers [101-109], by the association of natural materials with a synthetic polymer [20, 23, 44, 45, 51, 54-56, 59, 76, 110-128], by association of others materials [24, 30, 34, 41, 53, 129-132]. When analyzing table 6, it is clear that most biomaterials succeed (to a higher or less extent) on regenerating hyaline matrix, while delivering bioactive agents such as cells and/or growth factors, as well as fulfilling fundamental requirements for translation into human scenario. Major limitations of these gels/hydrogels relate to unsatisfactory mechanical properties, capable to immediately withstand load after treatment, as well as a mismatch of biomaterial degradation rate as compared to tissue regeneration (either too fast or to slow). The combination of the hydrogel with a rigid scaffold has been tested (for example PLCL[23], PLA/ PLGA[92], aiming to improve mechanical properties, whereas the downside relates to loss of injectability, and consequently, adequacy of the system to be delivered by a minimally invasive approach. Crosslinking mechanisms differ among the biomaterial types, yet can be used, to a certain extent, to fine-tune mechanical properties as well as degradation rate of the hydrogels. Not less important in the cartilage repair equation, is the capacity to mimic the complex layered structure of articular cartilage tissue. Although current gels and hydrogels are still limited in this regard, future developments in the biomaterials field might pursue this target, by providing more sophisticated, smart and multifunctional materials for improved cartilage regeneration[133, 134].

Table 6 - *Characteristics of the biomaterials used as gels / hydrogels for cartilage repair.*

		Injectability	Delivery of bioactive agents	Adverse immune response	Cellular recognition	Pathogen transmition	Crosslinking	Mechanical properties	Degradation	Integration with native tissue	Hyaline cartilage	Ref
NATURAL	**collagen**	+	+	-	+	-	+	-	-	+-	+-	21-22, 25-26, 28, 32, 39, 47, 50, 52, 58, 63-64, 69-74
	alginate	+	+	+	-	+	+	-	-	+-	+-	37-38, 40, 48, 62, 75-78
	fibrin	+	+	+	+	+	+	-	+	+	+	29, 33, 79-80
	platelet-rich plasma	+	-	+	+	+	+	-	-	+	+	61, 81
	hyaluronic acid	+	+	+	+	+	+	-	-	+-	+-	27, 31, 57, 82-83
	gellan gum	+	+	+	-	+	+	+	+	+	+	84
	chitosan	+	+	+	+	+	+	+	+	+-	+	42, 85
SYNTHETIC	**oligo(poly(ethylene glycol) fumarate)**	+	+	+	+	+	+	+	+	+	+-	43, 46, 87
	poly(N-isopropylacrylamide-co-acrylic acid)	+	+	+	+	+	+	+	/	+	+	88
	silanized hydroxypropyl methylcellulose	+	+	+	+	+	+	+	/	/	+	36
	polypeptides	+	+	+	+	+	+	-	-	+	+	35, 65, 91

Label: + : positive for cartilage repair; - : negative for cartilage repair; / : lack of information

Regarding the animal model used, the rabbit was the preferred, comprising 73,3% of all studies. Rabbits gather several features that make it an attractive model for cartilage regeneration research. It is of easy handling, caging and care, has a good cost effectiveness and enough dimensions of the trochlear groove and condyles for the induction of a 3 to 4 mm diameter cartilage defects [135]. However, the relatively thin cartilage thickness (approximately 0.4 ± 0.1 mm in the trochlear groove) [136], has limited the volume size of the cartilage defect [135]. Another limitation of this animal model is the high degree of the rabbit knee flexion, creating a partial weight-bearing condition when the trochlear groove is chosen as location for cartilage defect induction/repair [137]. The present review revealed that the majority of studies used were immature rabbits younger than 8 months, the minimum age considered for maturity of rabbits [138]. The above-mentioned disadvantages and the high potential of the rabbit model for spontaneous healing [84, 139], especially in immature animals, are important limitations to address when the rabbit is used as a translational model to human knee cartilage. Herein, it was noticed a progressive use of larger and more weighted animal models, allowing bigger cartilage defects that reproduce better the size,

depth and conditions of human cartilage lesions [135]. Furthermore, some of these models, as opposed to rabbit, have a low spontaneous cartilage repair ability [140-142] and similar to humans, suffer from osteochondritis dissecans and osteoarthritis pathologies [135].

An articular cartilage defect is classified as full or partial-thickness defect according to the penetration into the subchondral bone [116, 143]. Considering the known cartilage thickness of the different animal models [135, 144], the majority of the defects overviewed in this review are deeper than the expected cartilage thickness for those models, therefore, these were classified as full-thickness defects or as osteochondral defect. This is a very important drawback regarding the relevance of the models used for evaluation of cartilage repair performance, given that in humans, superficial cartilage lesions are the most common, and only 5% are osteochondral defects [3].

Most of the studies have reported the treatment of cartilage defects at an acute stage. From the total publications analyzed, only 6 were related to chronic stages of the cartilage defect [34, 63, 64, 75, 111, 113]. It is recognized that a chronic cartilage lesion is a diverse condition as compared to an acute cartilage lesion [26, 34, 145]. This fact highlights the importance of addressing the correct stage of lesion progression in animal models when translating to human treatment.

As for tissue characterization, the majority of the studies included immunohistochemical evaluation of the neo-cartilage by evaluating the presence of collagen type I and type II, whereas expression of type X was determined in only 3 studies [19, 115, 132]. It is important to identify the expression of collagen type X, in order to exclude the possibility of hypertrophic tissue development or a transient cartilage [64].

Determination of gene expression was performed in 24 studies. In these, an increase in cartilage-related gene expression was found in the regenerated tissue. Nevertheless, given the mismatch of information regarding gene expression, it is not possible to perform a full comparison between studies.

Assessment of mechanical performance of the new tissue is a relevant dimension when evaluating quality of the cartilage repair. Yet, its implementation is difficult, as it depends on anatomical location, measurement methodology and specific conditions of the joint [66]. The mechanical properties of the repaired tissue were evaluated in only 8 studies. In most of these, properties of the new tissue were similar or close to native cartilage [24, 32, 34, 77, 114]. Some authors found inconsistent results [75] and repaired tissue showed a higher stiffness as compared to normal cartilage [24, 94]. As expected, similar mechanical properties between repaired tissue and normal cartilage was correlated with regenerated hyaline-like cartilage, except for the study by Pulkkinen

et al. [32], which despite mechanical properties being similar to native cartilage, the repaired tissue did not correspond histologically to hyaline-like cartilage. From these studies, two main issues can be highlighted: (i) large variety of reported methodologies among studies for determining mechanical performance of regenerated cartilage; (ii) adopted methodologies that do not reflect natural physiological condition [66]. These issues pose additional challenges when assessing quality of the regenerated cartilage in animal models using new biomaterial/therapeutic candidates, and when translating such performance during proof-of-concept or pre-clinical setting, to human performance in clinical setting.

The majority of the studies did not compare the treatment groups with reference treatments, adopted as clinical standard, such as microfracture or osteochondral grafting, which would be of high value to infer the relative efficacy of the new biomaterial/therapeutic candidates. For full-thickness defects (the most frequent defect type studied), non-treated control group acts in a similar way to microfracture as there is exposure to bone marrow. Yet, for partial-thickness defect, only 1 study compared the outcome with microfracture treatment [86]. Concerning osteochondral grafting, only 2 studies compared the results of between scaffold treated groups with osteochondral grafting [34, 48].

Regarding the use of cells, most studies used chondrocytes, although mesenchymal stem cells (MSC) have been also highly explored [39]. Adipose mesenchymal stem cells (aMSC), muscle mesenchymal stem cells (mMSC), synovium mesenchymal stem cells (sMSC) and bone-marrow mesenchymal stem cells (bmMSC) were used, which avoid donor site morbidity in the cartilage tissue. Among the different stem cell sources, it was stated that sMSC and bmMSC show a greater chondrogenic potential as compared to aMSC or mMSC, while one study reported, in addition, greater proliferation potential of sMSC[28]. Many researchers have reported an improvement in bone and cartilage formation [39, 59] when MSC were implanted. These improvements were promising, with a superior cartilage bonding to adjacent native cartilage, when compared with articular chondrocytes [64]. However, some authors [44, 50, 54, 121] did not find better results in cartilage regeneration when MSCs were used.

Regarding the use of growth factors, a relationship between use of growth factors and inflammatory response or pathological changes, was not found. However, reported responses were identified in only 5 experiments that have used growth factors [24, 31, 62, 63, 65]. For 1 case using BMP-2, extensive ectopic bone formation was observed [31].

TGF–β seems to be dose-dependent and lower concentrations are more effective in repairing cartilage defects and decrease osteophyte formation [62]. TGF – β1 has been suggested to have a pro-inflammatory response, but no study using TGF – β1 reported an inflammatory response. TGF–β1 promoted trabecular bone subchondral appearance but did not improve cartilage cell morphology or glycosaminoglycan (GAG) expression[44], while TGF – β3 was suggested to have a chemotactic cue for cell homing [114]. The combination of BMP-7 and TGF–β1 was found to induce chondrogenic differentiation [115].

To be considered mature hyaline cartilage, the repaired tissue must exhibit normal morphology of chondrocytes and normal safranin O staining and possess an adequate structural organization with vertical columnar alignment of chondrocytes. When the last condition is not attained the repair tissue is classified as immature. If the tissue is composed of dense spindle-shaped fibroblasts, the tissue is graded as fibrous tissue. When the repair tissue contains cells beginning to differentiate toward chondrocytes, the tissue is called as undifferentiated mesenchymal tissue [48, 146]. Another important aspect is that 3 studies did not obtain statistically significant improvement in treated groups when compared with untreated group [30-32]. Although the majority of studies reported improvement of cartilage regeneration in treated groups, 22 studies did not recognize formation of hyaline-like cartilage at the repaired defect site. Therefore, better scoring of repaired tissue does not mean necessarily hyaline-like cartilage formation. Further discussion might focus on reliability and adequacy of scores used to evaluate regenerated cartilage tissue. Bonasia et al., tested the inter- and intraobserver reliability of 10 scores and concluded that, for evaluation of cartilage repair in animal models, the ICRS II, O'Driscoll and Modified O'Driscoll scores are preferential given their high reliability, and the fact that the whole joint is available for histological assessment[147]. On the other hand, for evaluation of human cartilage biopsies the ICRS I or II or Oswestry score are preferable given the limited tissue available[147].

The studies analyzed herein evaluated repaired tissue mostly by the O'Driscoll score, followed by the Wakitani. One of the limitations of these scores relate to the lack of validation by biochemical analysis [148]. Only the Bern score has undergone such validation, yet has been considered more adequate for analysis of tissue-engineered constructs[148] instead of repair of cartilage in animal models. Accordingly, it was not used in any of the revised studies. For the O' Driscoll score, safranin O staining grading is not reflected in the final score and a limited difference was observed between a "moderate" and a "poor" quality of regenerated cartilage[148]. Although O' Driscoll score

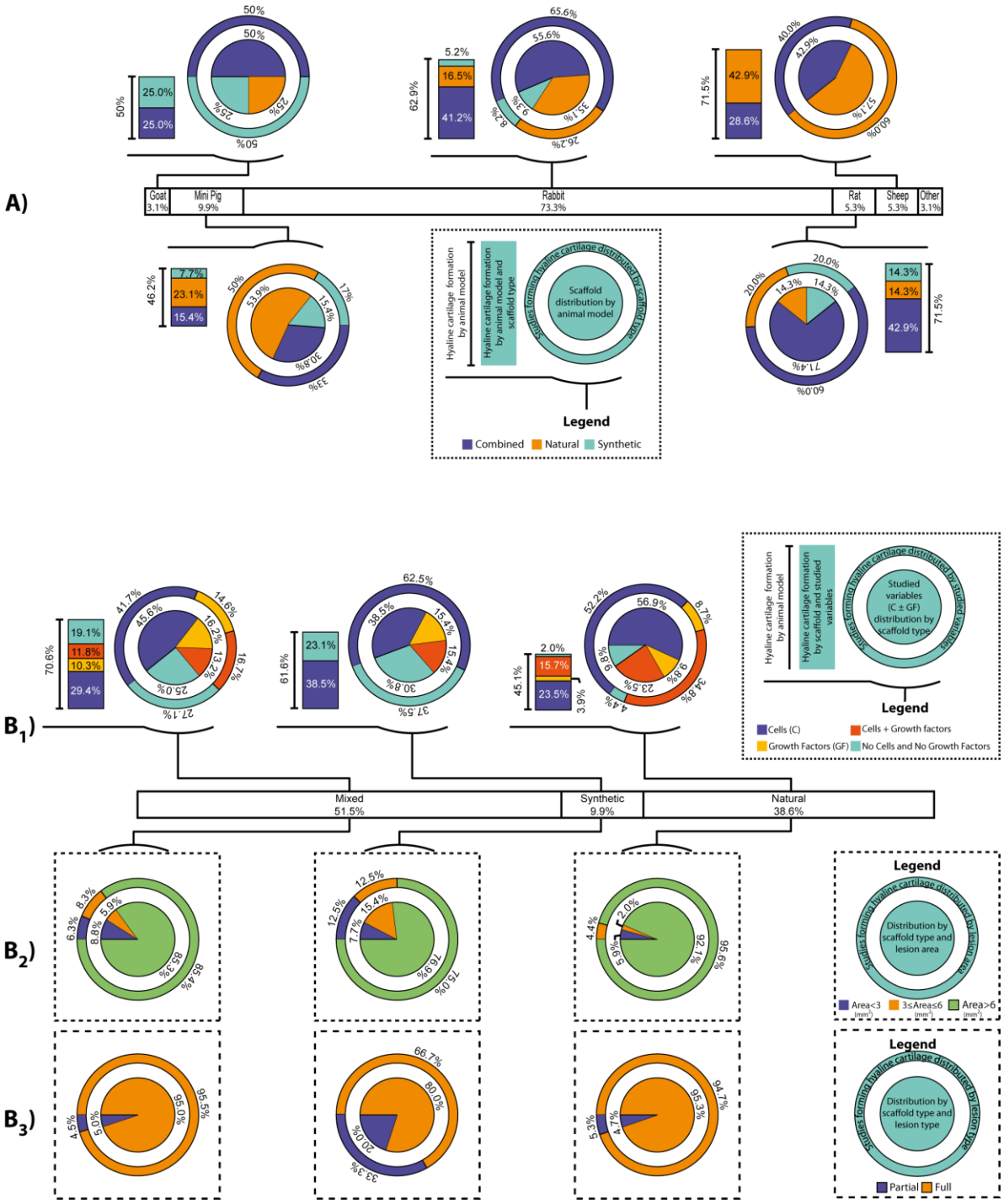


Figure 4 - *Correlation of data variables.* ***(A)*** *Inner circle: distribution of scaffold type used in each animal model; Outer circle: efficacy of each scaffold type in forming hyaline cartilage. Lateral column displays overall efficacy of the animal model in yielding hyaline cartilage outcomes, further discriminated by scaffold type.* ***(B1)*** *Inner circle: distribution of cell ± growth factors used in each scaffold type; Outer circle: efficacy of each cell ± growth factor combination in forming hyaline cartilage. Lateral column displays overall efficacy of scaffold type in yielding hyaline cartilage outcomes, further discriminated by cell ± growth factor combination.* ***(B2)*** *Inner circle: distribution of lesion area used in each scaffold type; Outer circle: efficacy of lesion area in yielding hyaline cartilage outcomes.* ***(B3)*** *Inner circle: distribution of lesion type used in each scaffold type; Outer circle: efficacy of lesion type in yielding hyaline cartilage outcomes.*

includes evaluation of repaired tissue structure, it does not consider other parameters such as mineral degeneration, vascularity, subchondral bone, viability cell population, inflammation and

cartilage plug quality[148]. As previously reported[149], evaluation of cartilage repair should make use of more than one score, complemented by biochemical, automated histomorphometry and biomechanical correlation.

Data Correlation

Given the above-mentioned compilation of data, one would be tempted to understand which combination of factors would seem the most promising in yielding regeneration of cartilage tissue. Despite the high number of variables and possible combinations, an excel VBA application was developed in order to correlate data. Studies were characterized as "hyaline" or "no hyaline" based on the studies' author classification of repaired tissue. Subsequently, studies were selected based on the use or no use of cells (C) and/or growth factors (GF), by animal model or lesion size, ultimately correlated by type of scaffold (natural, synthetic or combined materials). Outcomes are displayed in figure 4.

Regarding the animal model (Fig. 4A), despite the rabbit not being recommended as a model to evaluate cartilage repair due to small cartilage thickness, and high spontaneous regeneration [150, 151], when analyzing Fig. 4A, it is evident that among all animal models, the rabbit has been the most widely used, comprising 73.3% of all studies. Of these, 62.9% claimed to have generated hyaline-like cartilage tissue. Apparently, combined scaffolds were responsible for such outcome, comprising 41.2% of the hyaline repaired tissue. Nevertheless, for bigger animal models (goat, sheep and mini pig), the natural origin scaffolds seem to result in superior hyaline-like cartilage regeneration, as compared to those using combined or synthetic hydrogels.

When analyzing from another perspective, it was possible to determine that 55.6% of all rabbit studies used combined scaffolds, and these generated 65.6% of all hyaline-positive outcomes. This trend is maintained for all animal models, whereas the synthetic scaffolds seem to yield inferior outcomes.

Figure 4B1 displays an analysis of the combination of cells (C) and/or growth factors (GF) with the different types of hydrogels, and their synergistic effect on cartilage repair. In fact, of all studies analyzed, 51.5 % used hydrogels composed of combined materials and resulted in a 70.6% success rate on generating hyaline-like cartilage. Those using scaffolds of natural origin (38.6%) seem less successful, where only 45.1% generated hyaline regeneration. Nevertheless, it does seem that the presence of cells generally improve probability of successful regeneration of tissue, as major

percentage of successful outcomes where achieved through the use of cells in combination with the scaffold, while the positive effect of the presence of growth factors is not so evident (Fig. 4B1). An additional correlation factor was lesion size, where type of scaffold (natural, synthetic or combined), was related to the lesion area (<3 mm^2, 3-6 mm^2 or >6mm^2) and relative percentage of incidence on generating hyaline cartilage was analyzed (Fig. 4B2). Overall, hydrogels of combined materials seem to perform better than natural or synthetic hydrogels, in nearly all dimension ranges, yet it seems that for larger lesions, natural origin hydrogels provided better outcomes.

On what regards deepness of lesion (Fig. 4B3), full-thickness was the most used and the most successful in obtaining hyaline cartilage, according to their authors. However, interpretation of this outcome is limited to the reduced number of studies that have tested repair of partial lesions (only 5% of all studies).

Study Limitations Acknowledged by Authors

Some authors pointed several limitations in their studies: the dimension of the sample [27, 30, 34, 39, 43, 67, 103, 108] and specific problems with design of the study [44, 65, 96, 101, 102]. In addition, several limitations have been pointed out, such as lack of biomechanical evaluation [28, 67, 99, 104-106, 112, 129, 131], short follow-up [27, 43, 62, 77, 101, 103, 105, 106, 109, 113, 122, 130, 131], animal immaturity and type of animal model [77, 104, 105, 110, 117], poor representativeness of human pathology [93], origin-cell identification not possible in the majority of the studies [25, 26, 67, 131], absence of a specific rehabilitation program [113], and experimentation under no load bearing conditions [34, 106]. The International Cartilage Repair Society (ICRS)[150] and the American Society for Testing and Materials (ASTM)[151] have published guidelines and recommendations for preclinical studies aiming cartilage repair, that could be considered by researchers in order to generate valuable and comparable data, ultimately contributing to stronger advancement of knowledge in the field of cartilage repair.

Conclusions

In summary, hydrogel biomaterials seem to be promising candidates for cartilage repair, given that hyaline-like cartilage development was proved in a considerable number of studies. A potential advantage of using hydrogels for cartilage repair is its suitability for arthroscopic delivery, yet, in

many studies, hydrogel properties did not seem compatible with this minimally invasive approach. Overall, further development on surgical technique will be required.

The majority of the published papers addressed small, acute and a full-thickness cartilage defect in a non-weight bearing area. These conditions are very different from those found in human patients which is a concerning limitation considering translation of experimental learnings towards human treatment. The need for animal models and experimental designs that consider those aspects is obvious and must be considered in future animal experimentation studies.

In addition, anticipation of potential therapeutic efficacy in human demands a more conclusive mechanical evaluation of the regenerated tissue, as well as long-term studies. Not less important is the need of standardization of the evaluation procedures, especially on what concerns histology in order to enable comparison among different studies. The use of uniform guidelines for the definition of the general conditions and techniques to be used in cartilage repair experiments is mandatory to ensure comparability of studies.

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SECTION III

CHAPTER V

In Vitro and *in Vivo* Performance of Methacrylated Gellan Gum Hydrogel Formulations for Cartilage Repair

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ABSTRACT

Methacrylated gellan gum (GGMA) formulation is proposed as a second-generation hydrogel for controlled delivery of cartilage-forming cells into focal chondral lesions, allowing immediate in situ retention of cells and 3D filling of lesion volume, such approach deemed compatible with an arthroscopic procedure. Formulation optimization was carried out *in vitro* using chondrocytes and adipose mesenchymal stromal/stem cells (ASCs). A proof-of-concept *in vivo* study was conducted using a rabbit model with induced chondral lesions. Outcomes were compared with microfracture or non-treated control. Three grading scores were used to evaluate tissue repair after 8 weeks by macroscopic, histological and immunohistochemical analysis. Intense collagen type II and low collagen type I gene and protein expression were achieved *in vitro* by the ASC+GGMA formulation, in light with development of healthy chondral tissue. *In vivo*, this formulation promoted significantly superior de novo cartilage formation compared with the non-treated group. Maintenance of chondral height and integration with native tissue was further accomplished. The physicochemical properties of the proposed GGMA hydrogel exhibited highly favorable characteristics and biological performance both *in vitro* and *in vivo*, positioning itself as an attractive xeno-free biomaterial to be used with chondrogenic cells for a cost-effective treatment of focal chondral lesions.

Key Words

cartilage repair

methacrylated gellan gum

hydrogel

adipose stromal/stem cells

chondrocytes

INTRODUCTION

Complementary efforts for improving cartilage repair[1] have addressed optimization of combination strategies involving predominantly autologous articular chondrocytes[2, 3] for which decades of performance history have been collected[4-7]. Robust long-term outcomes is among its major advantages[8, 9], yet suboptimal cell retention within cartilage lesion sites has been a concern[10-12] which has driven the development and marketing of novel scaffolds or matrices to enhance efficacy of these procedures[12-14] Nonetheless, most surgical protocols intervene directly on the subchondral bone either for recruitment of cells or for fixation of the scaffold, involve additional fixation systems, or require invasive procedures, such as arthrotomy, to be effectively implanted[15, 16].

In previous works[17-19] gellan gum (GG) has been proposed as new biomaterial for cartilage tissue engineering applications. Its versatility and efficacy has been demonstrated for cartilage repair strategies involving both subchondral stimulation and cell transplantation using a rabbit model[18]. Both histological and gene expression outcomes confirmed the potential of this approach for cartilage repair but limitations concerning usability and crosslinking kinetics have been identified, which could limit is translation into a clinical setting. Subsequent work explored alternative synthetic routes to enhance performance of GG hydrogels, namely the methacrylation of the molecula (GGMA) [20-22] for other biomedical applications. Rational design modification of GG yielded a second-generation GGMA polymer endowed with improved physicochemical characteristics, including better solubilization, liquid formulation prior to injection at room temperature, improved gelification kinetics, and more robust mechanical properties of the hydrogel [20-22] the latter being greatly dependent on the crosslinking mechanism. In an applied perspective, the adoption of an injectable formulation based in GGMA in the context of cartilage repair is highly attractive, as its solution-state properties make it potentially compatible with minimally invasive procedures.

Given the positive track-record of the parent GG molecule on what regards safety and performance, it becomes mandatory to quantify the actual benefits of GGMA in the cartilage repair application context. Understanding physicochemical performance of GGMA could be explored to simplify the surgical protocol, to improve delivery and functional commitment of cells employed, as well as to minimize damage of the subchondral compartment during the surgical protocol. In this regard, this study aims to comparatively assess the safety and performance of GG and GGMA by *in vitro* methods, as well as to characterize the performance of GGMA hydrogel as vehicle for delivery and

retention of chondrogenic cells within chondral lesions, by assessing functional development of hyaline cartilage tissue in a rabbit model.

On the perspective of functional performance of cells, the risk of chondrocyte de-differentiation[23], or lack of potency of the autologous chondrocytes[24, 25][26] along the need for double surgery and prolonged surgical pre-planning, has inspired the study of alternative cell sources, including mesenchymal stromal/ stem cells (MSC) in general, and adipose-derived stromal/ stem cells (ASC) in particular [17, 18, 27]. As compared to other MSC sources, adipose tissue can be harvested with reduced morbidity at the donor site and yields of ASC are considerably high.[28] The immunomodulatory and anti-inflammatory properties of ASC makes them an especially attractive cell source for development of off the shelf regenerative medicine treatments[27, 29, 30].

Herein, preliminary screening demonstrated improved cell viability of ASC within ionic-crosslinked GGMA as compared to photo-crosslinked, therefore favoring further experimentation with ionically crosslinked GGMA. The best performing combination was further evaluated for the treatment of focal chondral lesions in a rabbit model, by adopting a physiologically-inspired crosslinking approach devoided of toxic photo-initiators and electromagnetic radiation sources, which is highly desirable from both a regulatory and surgical protocol perspectives.

MATERIALS AND METHODS

In vitro chondrogenesis

Preparation of Purified GG and GGMA

Commercial GG (GGc) (GelzanTM, Sigma-Aldrich) was purified according to the method described by Doner[31] with several modifications. Briefly, GGc was suspended in distilled water (1% w/V) and warmed to 60 °C with stirring. To this solution was added Amberlite IR-120 (H+ form) (Sigma-Aldrich) until pH 2.5. The suspension was filtered and aqueous sodium hydroxide (NaOH, 1 N) was added until pH 8, while stirring. The resulting solution was filtered and the filtrate poured onto absolute ethanol (1 L), forming a thick fibrous precipitate. After 1 hour, the precipitate was filtered, washed with absolute ethanol and dissolved in distilled water. The resulting solution was transferred to a cellulose membrane (Molecular weight cut-off (MWCO)12 KDa) and dialyzed against distilled water for 3 days. After freezing (-20 °C) and lyophilization, the purified GG (GGp) was obtained. GGMA, with a degree of substitution with methacrylate groups between 1.5 to 5% was prepared as follows: GGc was dissolved in water to give a solution of 1% w/V concentration. Heating was stopped

and the solution pH was adjusted to 8.5 by NaOH (1 N). Thereupon, excess glycidyl methacrylate was added in one portion and the methacrylation reaction was allowed to proceed for 24 h whilst maintaining the solution pH close to 8.5. Acetone was then added to the reaction mixture which was allowed to stand for 2 hours. The precipitate was recovered by filtration, dissolved in distilled water and then placed in a cellulose dialysis membrane (MWCO 12 KDa) and dialysed against distilled water for 7 days. The dialyzed solution was then frozen at – 20 °C and subsequently freeze-dried to give GGMA as an amorphous white solid. For hydrogel preparation, GGp and GGMA powder were dissolved in deionized water to achieve solutions at 1.25% w/V and 2.5% w/V, respectively. Dissolution was effected at 37 °C in a water-bath with 100 rpm agitation.

In Vitro Culture of Human Cells

Human nasal cartilage (hNC) was obtained with informed consent, as surgical waste from a local hospital and further processed for isolation of chondrocytes as described elsewhere[17]. Chondrocytes where thawed and expanded in DMEM:F12, supplemented with 10% v/v FBS and 1% v/v antibiotic-antimycotic (Gibco, USA) until passage 3. Human adipose tissue (hAT) was obtained from liposuction procedures, after informed consent and medical questionnaire according to European directives. Collection of adipose samples was approved by the Institutional Review Board of "Centro Hospitalar de São João", Portugal. Briefly, hAT was washed with a decontamination solution (Base-128 Alchimia, Italy) and digested with collagenase (0.4 U/mL, NB6, SERVA, Germany) for 1 h at 37 °C with agitation. The stromal vascular fraction (SVF) was collected after purification steps that include washing, centrifugation and lysis of red blood cells. Human adipose mesenchymal stromal/stem cells (hASC) were obtained from SVF by plating and further expansion in low serum media (MesenPro, Gibco, USA) or xeno-free media (Fibrolife, Lifeline, USA) until passage 2 or 4. Quality control included validation of MSC immunophenotype (CD31, CD34, CD45, CD73, CD90 and CD105, BD Biosciences, USA) characterized by flow cytometry analysis (FACS Canto, FACSDiva software, antibodies BD Biosciences, USA), and trilineage differentiation (StemPro, Gibco, USA) identified by alizarin red, oil red O and alcian blue staining's for osteogenesis, adipogenesis and chondrogenesis, respectively.

Preparation of Cell-Encapsulated Hydrogels for In Vitro Culture

An initial comparison study was performed to evaluate comparative performance of GG and GGMA with respect to hydrogel formation and metabolic activity of encapsulated hASC. Selected

formulations were further used for assessment of chondrogenesis by hASC or hNC (Fig. 1). Cell suspensions were prepared in cell culture media and mixed with GG or GGMA solution in a 2:8 ratio in order to yield a final cell density of $5x10^6$ cells/mL and hydrogel concentrations of 1 and 2% w/V, respectively. Cellular hydrogels of 20 µL volume were pipetted (with aid of a positive displacement pipette) into wells of non-adherent cell culture well-plates and covered with culture media for crosslinking. To induce chondrogenesis of hASC, serum-free chondrogenic media (StemPro, Gibco, USA) was used. At specific time-points (i.e. 0, 7, 21 days), individual hydrogels were collected for analysis. The hASC-GGMA combination was further scaled to hydrogels of 50 µL volume containing $10x10^6$ cells/mL and tested for chondrogenesis.

Assessment of in vitro cell viability and chondrogenesis

Cell metabolic activity was determined at each time-point by MTS assay (Promega USA) and cell viability was further microscopically assessed by Live / Dead assay (calcein AM and propidium iodide [Invitrogen, USA 1 mg/mL]). For histology and immunohistochemistry (IHC), hydrogels were fixed (10% formalin), followed paraffin processing. Histochemical staining of glycosaminoglycans (GAGs) by Safranin O/ Fast green and Alcian Blue were performed as previously described in [17]. For IHC, reagents from Vector Laboratories (UK) were used. Sections were incubated into recommended antigen retrieval solutions, followed by inhibition of endogenous peroxidases with 0.3% H_2O_2 and blocking with normal horse serum. Thereafter, sections were stained with primary antibody Mouse anti-human Anti-Collagen I or Mouse anti-human Anti-Collagen II (Abcam, UK) for 1 h, RT and a diluted biotinylated secondary antibody solution (VECTASTAIN® Elite ABC kit) for 30 min, RT. Signal development was performed with the DAB substrate kit. For gene expression analysis, hydrogels were collected into TRI Reagent (Sigma-Aldrich, USA) and recommended protocol for RNA extraction from tissues was followed. Complementary cDNA was obtained by using the High Capacity cDNA Reverse Transcription Kit. Gene amplification was conducted using TaqMan® Fast

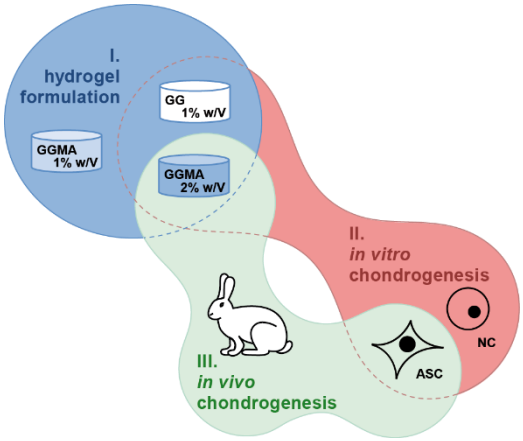

Figure 1 - *Schematic representation of experimental design.*
Hydrogel formulations based on gellan gum (GG) and its methacrylated derivative (GGMA) were tested for gelification and cell encapsulation (stage I). Two formulations were selected for in vitro assessment of chondrogenesis (stage II) using human chondrocytes (NC) and adipose mesenchymal stromal / stem cells (ASC). A final formulation was applied for treatment of focal chondral lesions in an induced rabbit model (stage III).

Advanced Master Mix and TaqMan™ Gene Expression Assays for Collagen type II (Hs00264051_m1) and Collagen type I (Hs00164004_m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Hs99999905_m1) was chosen as an invariant standard (housekeeping gene). Quantitative reverse transcription (RT-qPCR) analysis was carried out with the StepOnePlus™ Real-Time PCR System and software (all reagents and equipment from Applied Biosystems, USA). Results were normalized to GAPDH and expressed as relative gene expression using the ΔΔCt method. The expression data were presented as average values for each group (n = 3 ± SD).

In vivo chondrogenesis

Chondral Lesion Induction and Repair in a Rabbit Model

The ICRS and ASTM guidelines were followed for a proof-of-concept (PoC) study in rabbits to assess *in vivo* cartilage tissue repair[32, 33]. All animal procedures were based upon the "3R's" policy, approved by the Institutional Ethical Committee, according to the National authority Guide for the Care and Use of Laboratory Animals. Skeletally mature (12-14 weeks-old) New Zealand white rabbits (2.5 ± 0.25 kg; Charles-River, France, n=6) were used for harvesting adipose tissue and subsequent autologous treatment of focal chondral lesions. Interscapular adipose tissue (~10 g) was collected under anesthesia with a mixture of ketamine hydrochloride (Imalgene, 25 mg/kg i.m.) and medetomidine hydrochloride (Domitor, 0.3 mg/kg i.m.). The obtained adipose tissue samples were digested for 1 h at 37 °C, 100 rpm with Collagenase NB4 Standard Grade 0.2 U/mL (Serva, Germany). After complete digestion, cells were cultured in complete media based on alpha MEM supplemented with 10% v/v FBS and 1% v/v antibiotic-antimycotic (Gibco, USA), until passage 2. One week after adipose tissue harvest, surgery was conducted to create critical chondral defects in the knee for immediate administration of treatment. Rabbits were anesthetized as described above and both knees were shaved and disinfected. An internal para-patellar incision was made to expose the knee. The patella was dislocated, and two 4 mm-diameter lesions were made in the trochlear groove of each knee using a biopsy punch. Lesion sites were carefully cleaned with a curette to not affect the sub-chondral bone. Defects were randomly allocated to one of the following experimental conditions: i) rabbit autologous ASC encapsulated in GGMA hydrogel (GGMA+rASC); ii) microfracture (MFX) (positive control) and iii) empty lesion (negative control). Autologous rabbit ASC were encapsulated in GGMA hydrogels as described earlier ($10x10^6$ cells/mL, 2% w/V) immediately before delivery into the chondral defect. In situ crosslinking was promoted with PBS and a setting time of 10 minutes was allowed before closure. MFx were made

with a 0.8 mm Kirshner wire (6 holes per defect) with 1- to 2-mm depth from which bleeding was observed. Finally, the patella was reduced, and the wound was closed. After recovery from surgery, animals were placed in individual cages and fed ad libitum.

Assessment of In Vivo Cartilage Tissue Repair

Cartilage regeneration was allowed for eight weeks, after which animals were anesthetized as described earlier and euthanized (Eutasil, 200 mg/kg). In each knee, an internal para-patellar incision was made and the patella carefully dislocated. Macroscopic pictures were taken, and explant tissue was harvested with a 6 mm diameter punch in order to collect native tissue surrounding the lesion site, as well as subchondral bone. Explants were paraffin-processed after fixation (10% formalin) and decalcification (Biodec R, Bio-Optica, Italy). For IHC, sections were processed as described above, followed by incubation with primary antibody mouse anti-rabbit anti-collagen I (Abcam, UK) or mouse anti-rabbit anti-collagen II (Merck Millipore, USA) for 1 h, RT. Histochemical staining of GAGs was performed by Safranin O/ fast green and three scoring systems were used to assess the quality of cartilage repair, namely O'Driscoll, Pineda and Wakitani[34].

Statistical Analysis

Results are summarized by mean or median and corresponding standard deviation or interquartile range. For *in vitro* studies, Student's t-test and two-way analysis of variance (ANOVA) were used to evaluate differences among groups. Normality was evaluated by the Shapiro-Wilk test. When normality or homogeneity of variances was not verified, non-parametric tests were used. For *in vivo* studies, the histological scores for each specimen were evaluated independently by three observers at three different times. For evaluation, the observers were blinded for the type of treatment and the specimens were randomly allocated to each observer. The comparisons between treatment groups were performed by two-way ANOVA. Since there were no statistical differences between the observers' evaluations, the results were analyzed using one-way ANOVA. In cases where homogeneity of variances was not observed, the Kruskal-Wallis nonparametric test was adopted. Multiple comparisons were based on the Tukey HSD test or the Mann-Whitney test, with the corresponding significance level and Bonferroni correction. Statistical analysis was performed using the GraphPad Prism 4.0c software or IBM SPSS Statistics, version 23. Statistical significance was defined for $p<0.05$.

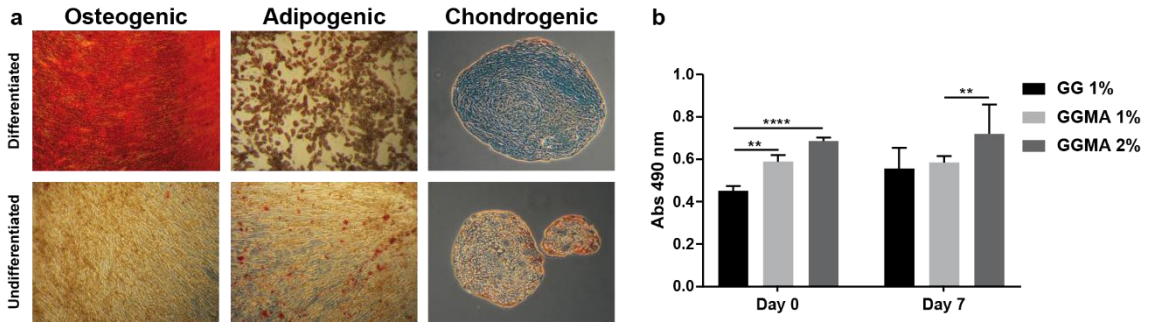

Figure 2 - *Preliminary in vitro studies with hASC**: a.** Trilineage differentiation of hASC identified by alizarin red, oil red O and alcian blue stainings for osteogenesis, adipogenesis and chondrogenesis, respectively. **b.** Metabolic activity of hASC encapsulated in gellan gum (GG) and methacrylated gellan gum (GGMA) hydrogel formulations (1 and 2 % w/V) upon encapsulation (day 0) and after 1 week in vitro culture. **p<0.01, ****p<0.0001.*

RESULTS

In vitro chondrogenesis

Trilineage differentiation capacity was confirmed for CD73+/CD90+/CD105+/CD31-/CD34-/CD45-hASC, with expressive mineralization, lipid formation and GAG deposition upon osteogenesis, adipogenesis and chondrogenesis, respectively (Fig. 2a). Upon encapsulation within GG-based hydrogel formulations, higher metabolic activity was observed for hASC encapsulated in GGMA 1% and 2% w/V as compared with GG at 1% w/V ($p<0.01$ and 0.001, respectively) (Fig. 2b). GG at 2% w/V provided inadequate sol-gel transition time for cell encapsulation studies. After 7 days of culture, highest metabolic activity was observed by cells encapsulated in GGMA 2% w/V ($p<0.01$) therefore this formulation was selected for further *in vitro* cell encapsulation studies concurrently with GG 1% w/V (Fig. 3).

After 21 days of *in vitro* culture, the viability of chondrocytes and hASC, assessed microscopically by live/dead assay, was comparable between GGp and GGMA hydrogels (Fig. 3a, top and middle rows). On the other hand, hASC chondrogenically differentiated within both hydrogel groups (hASC-chondro) demonstrated increased viability within the GGMA hydrogel (Fig. 3a, bottom row). On what regards expression of chondrogenic markers, both formulations favored maintenance of healthy chondrocytes as evidenced by significantly increased expression of collagen type II relative to collagen type I. Such response was superior by the GGMA formulation ($p<0.0001$) as compared with the GG ($p<0.001$). Furthermore, chondrocytes cultured within the GGMA hydrogel presented higher collagen type II expression ratio as compared with the parent GGp hydrogel (Fig. 3b, top) ($p<0.0001$). On what regards hASC (Fig. 3b, bottom), such cells effectively expressed collagen type II upon chondrogenic stimuli (21 days) when cultured within either hydrogel formulation ($p>0.05$).

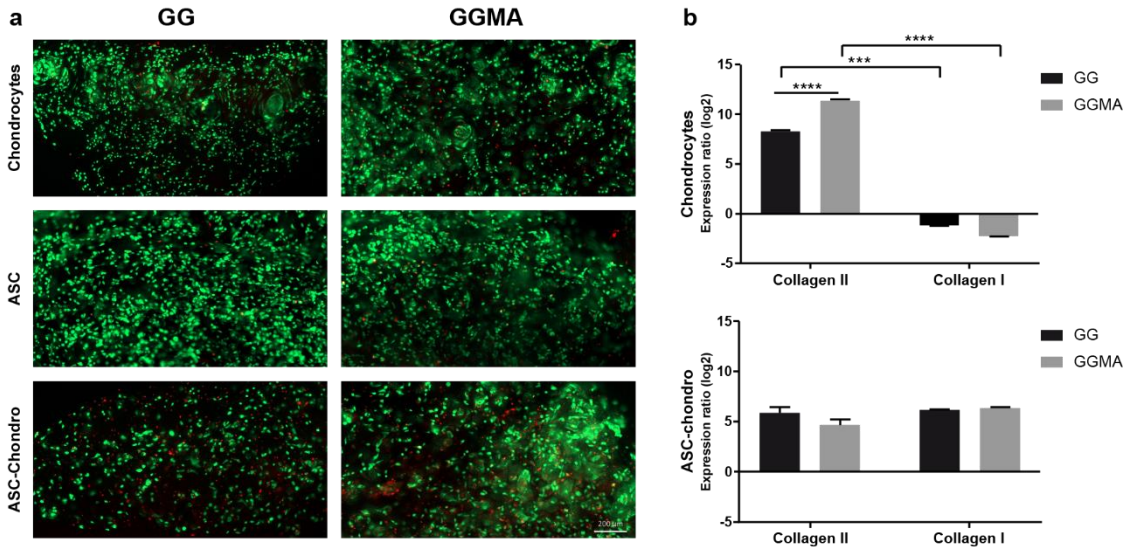

Figure 3 - *in vitro chondrogenesis**: a.** Microscopic imaging of encapsulated cells stained by calcein AM (live, green) and propidium iodide (dead, red) upon 21 days in vitro culture in 1 % w/V gellan gum (GG) and 2 % w/V methacrylated gellan gum (GGMA) hydrogels. **b.** Normalized gene expression ratio (day 21 to day 0) of GG / GGMA encapsulated chondrocytes (top) and chondrogenically induced hASC (bottom). ***p<0.001, ****p<0.0001.*

Concurrently, collagen type I expression was not superior to collagen type II at this time-point ($p>0.05$). When doubling hASC concentration within the GGMA hydrogel up to $10x10^6$ cells/mL, increasing expression of collagen type II was obtained in the course of chondrogenic differentiation (Fig. 4a). Simultaneously, very low expression of collagen type I was obtained along culture ($p<0.001$ and 0.05 at days 14 and 21, respectively). Samples were further collected for histological analysis and subsequent identification of extracellular matrix (ECM) components (Fig. 4b). Progressive deposition of healthy chondrogenic ECM was observed as evidenced by safranin O/ fast green staining of cartilage matrix, alcian blue detection of sulphated GAG and IHC of human collagen type II. The absence of collagen type I deposition also indicates development of non-fibrous cartilaginous tissue. Macroscopically, transparent hydrogels at the beginning of culture showed reduction in transparency (not quantified) into an off-white opaque appearance after 3 weeks *in vitro* culture (Fig. 4b, bottom row).

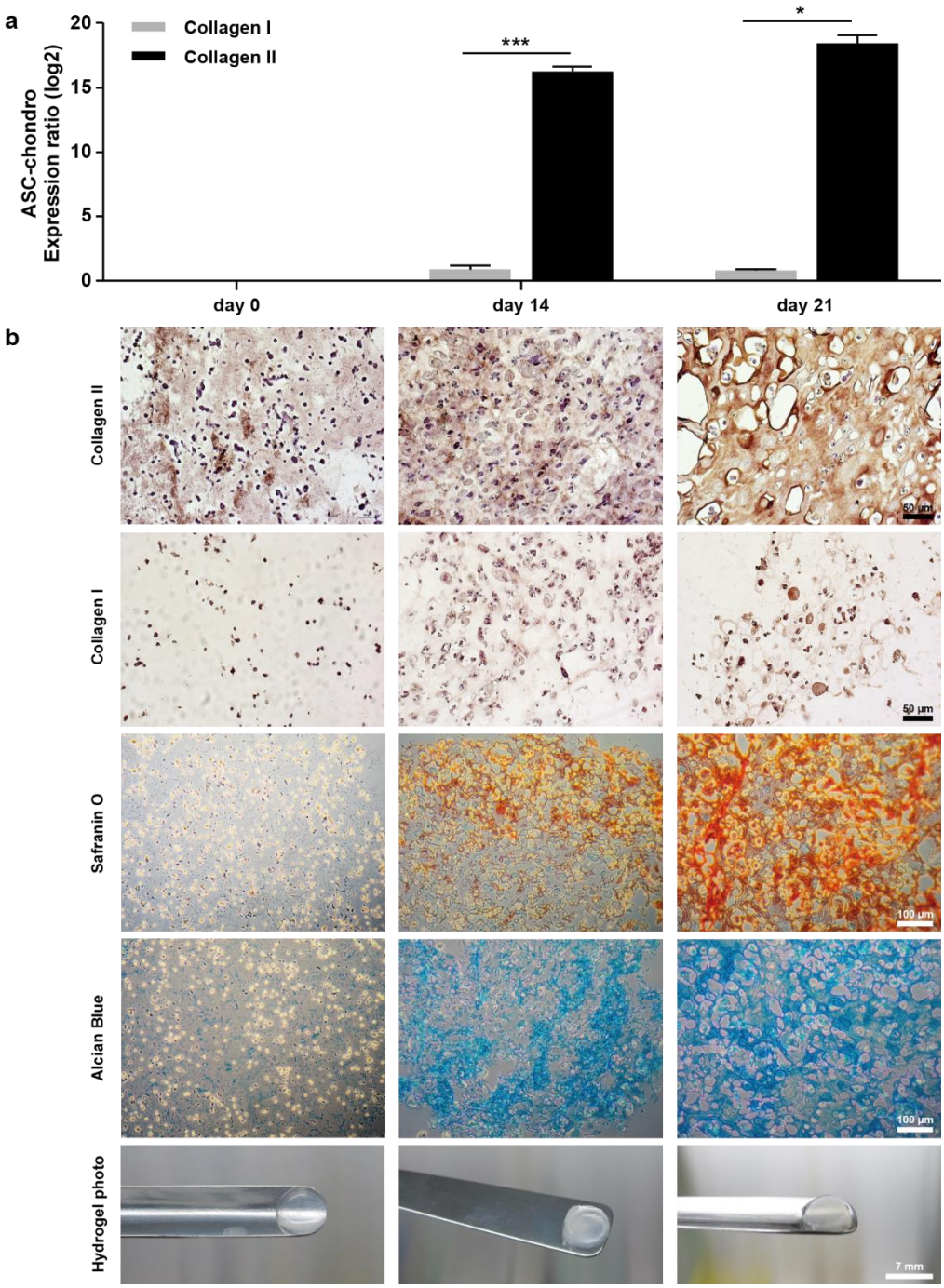

Figure 4 - *in vitro chondrogenesis of hASC encapsulated in GGMA 2% w/V:* ***a.*** *Gene expression ratio normalized to day 0. *p<0.05, ***p<0.001.* ***b.*** *Histological analysis and macroscopic imaging of hydrogel along in vitro culture.*

In vivo cartilage repair

At surgical treatment day, expanded rASC were mixed with GGMA solution at time of surgery so as to form a homogenous suspension. Upon injection into the lesion, the viscosity of the suspension

allowed spatial control of delivery within the lesion volume, without spill over at the edges of the defect. Gelification was allowed to occur during 10 min as to assure maintenance of the hydrogel in the lesion site, allowing immediate retention of cells in situ. Rabbits remained healthy during all experimentation period, presenting normal weight gain and absence of signs of infection or disease. From macroscopic observation at time of explant surgery, no apparent abnormalities of the patella position were observed, neither signs of inflammation, abnormalities of the synovium, loose bodies, osteophytes or degenerative process were found. Macroscopic observation showed native cartilage near the defect site as well as the opposing cartilage to be bright and white without visible degenerative signs. In all defects, tissue formation was observed (Fig. 5), and the margin between the defect and the surrounding cartilage were visible, which was more evident for the empty control group. The defects treated with the GGMA+rASC combination showed compact bright tissue filling, despite macroscopic variability observed between defects. The lesions treated by MF presented an irregular filling of the lesion site, with tissue of a dim appearance. A similar outcome was observed for untreated lesions (empty defects). Tissue explants were further harvested for histological analysis. The quality of cartilage repair was assessed by three scoring systems (Fig. 5), which have inverse scales for indication of cartilage quality and outcome: according to O'Driscoll, high point values indicate enhanced cartilage while according to Pineda and Wakitani, low total point values represents superior repair. Inter observer differences were assessed and no statistical differences were observed. Immunolocalization of rabbit collagen type II and collagen type I (Fig. 5) was performed to further characterize cartilage formed within the lesion site. Treatment of chondral lesions with GGMA+rASC (Fig. 5, top) allowed restoration of cartilage thickness, integration / bonding with native cartilage, as well as intense and reasonably homogenous staining of ECM throughout the lesion site. Quantitative assessment of repair by all three scoring systems indicates significant improvement in cartilage repair as compared to the untreated lesions ($p<0.001$). According to the O'Driscoll score, GGMA+rASC treatment also outperformed MFX ($p<0.05$). Lesions treated with MFX (Fig. 5, Middle) demonstrated overgrowth of the subchondral bone into the lesion site which was covered by a thin layer of cartilaginous matrix stained by safranin O and collagen type II. This layer is irregular and bonding with adjacent native cartilage is incomplete. Nevertheless, the extent of cartilage repair by microfracture was superior to untreated lesions, independently of the scoring system used ($p<0.05$).

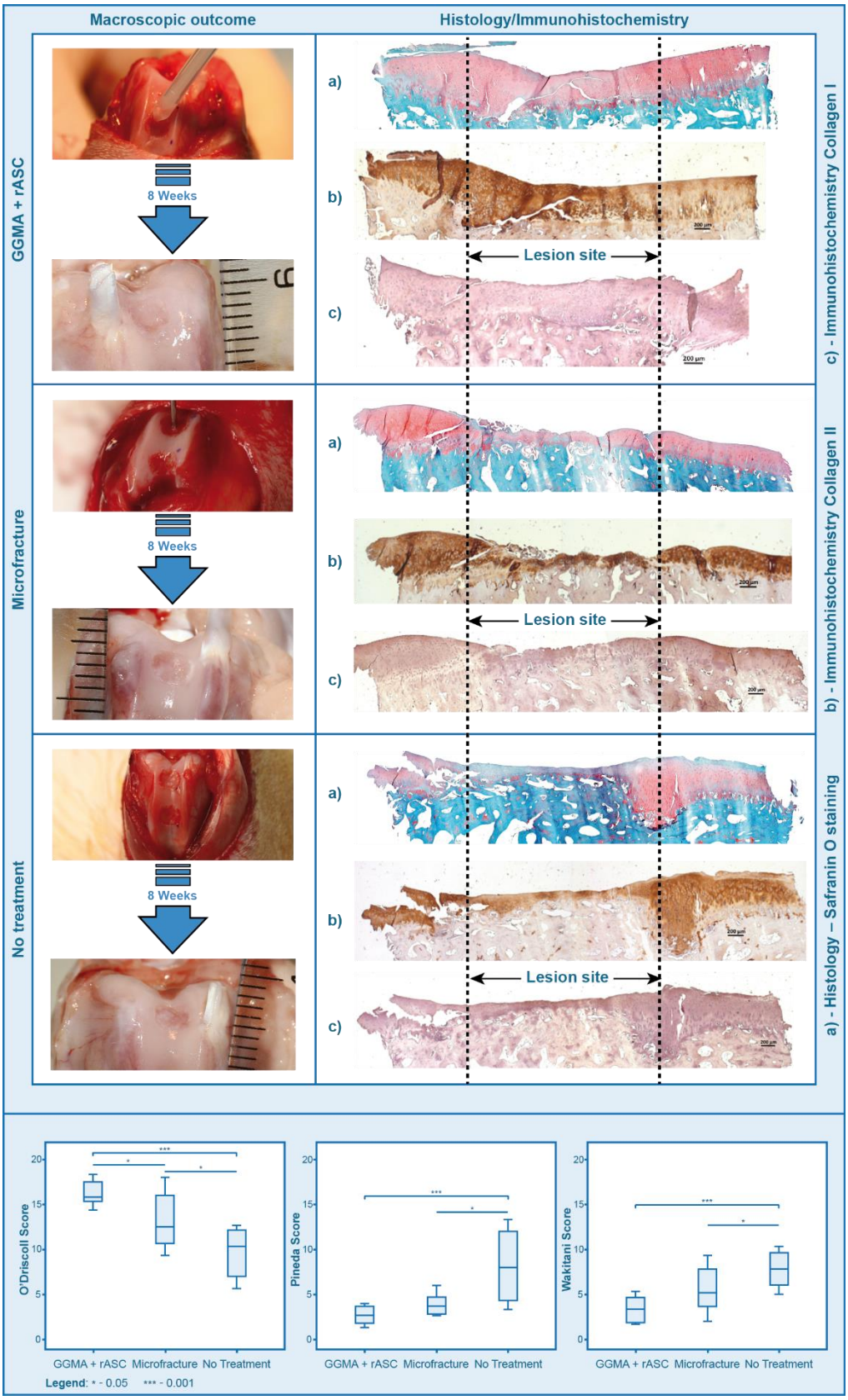

Figure 5 - *in vivo chondrogenesis of hASC encapsulated in GGMA 2 % w/V:* ***a.*** *Histological analysis and macroscopic imaging of experimental groups after 8 weeks of implantation.* ***b.*** *Histological scoring according to O'Driscoll, Pineda and Wakitani scores. *p<0.05, ***p<0.001.*

The bottom image represents histological assessment of untreated lesions, whereas overgrowth of subchondral bone was evident and covered by a thin regular tissue. Herein, very limited cartilaginous matrix was formed, as indicated by the lack of safranin O/ fast green and reduced collagen type II staining at the top layer of the tissue. Collagen type I deposition was negligible in all groups yet expressed slightly higher in the untreated defects.

Discussion

GG polysaccharide offers attractive features and characteristics for this particular application due to its aqueous solubility and viscous properties at physiological temperature which makes it appealing for implementation of cell combination and surgical implantation protocols. In addition, the crosslinking by physiological ions leads to formation of a stable, tridimensional structure and subsequent cell retention. In this regard, functionalization of the GG molecule by methacrylation extends solubility, allows control of spatiotemporal crosslinking, which combined extends flexibility of combination and implantation procedures and fine-tuning of hydrogel stiffness. For instance, at 2% w/V, GGMA presents increased storage modulus as compared with the unmodified polysaccharide, 89.5 ± 7.4 and 56.2 ± 1.4 kPa, respectively.[22] Matrix stiffness, as a result of increased concentration or biochemical cues, has been reported to influence stem cell fate and particularly chondrogenesis[35]. Within the context of this study, cells cultured within the GGMA 2% w/V hydrogel shown improved cell metabolic activity, viability, and healthy expression of chondrogenic markers as compared to the least concentrated (1% w/V) or non-functionalized matrix.

PoC in the rabbit model allowed evaluation of the cartilage repair potential of the GGMA 2% w/V hydrogel as compared to MFX treatment. Currently considered as a gold-standard treatment, MFX still has limitations on what concerns the quality of the regenerated tissue, which can ultimately lead to treatment failure upon recurrence of symptoms[36, 37]. In this study, MFX group outcome showed formation of a thin layer of chondral tissue concurrent with subchondral bone overgrowth and an irregular surface. Such repair outcome is likely to disfavor adequate load bearing as well as smooth, pain-free joint motion[14, 38, 39]. A different outcome was observed for lesions treated with GGMA-rASC – cartilage thickness was maintained equivalently to adjacent native tissue while avoiding bone overgrowth. A smooth chondral surface was obtained following this treatment and

ECM staining demonstrated reasonably uniform distribution of collagen type II and GAGs (Fig. 5). This repair outcome is believed to support long-term quality of the tissue as opposed to MFX treatment. At the 8 week time-point, statistical differences were obtained between these groups according to O'Driscoll scoring. Nevertheless, it is important to mention that quantitative scores with a broad numerical range such as the O'Driscoll system may increase the likelihood of finding statistically significant differences[34]. Still, the adoption of O'Driscoll score in the context of this study is pertinent as, contrary to alternative scores, it assesses integration of the repair tissue with its surroundings[34]. In this study, no additional fixation technique was used to retain the hydrogel within the lesion site and precise volume filling was achieved, avoiding the need for on-site shaping of the scaffolding structure, which could be an advantage as compared to other cell-based and tissue engineered cartilage products currently in clinical development[12]. In addition, viscous and sol-gel transition properties of the tested GGMA 2% w/V hydrogel allowed controlled delivery of the matrix containing autologous ASC [14], directly to lesion site, which favored delivery and retention of cells in situ. This fact is of significant importance as cell retention at lesion site is one of the main indicators of success for lesion repair[12, 40]. Adoption of the rabbit animal model for cartilage repair studies has significant advantages due to availability, ease of handling, low cost and abundance of comparative literature[14, 41], but poses challenges related to reduced thickness (0.4 ± 0.1 mm in the trochlear groove) and surface area of articular cartilage. This model is adequate for PoC studies during early stage development of new technologies, particularly on what concerns evaluation of fixation of implantable devices[32, 33]. In this regard, the fixation merits of GGMA-rASC combination was demonstrated in a particularly challenging environment. Heterogeneous outcomes were naturally observed yet bona fide chondral repair was obtained with this treatment group, which is attributed to GGMA-rASC combination alone. The analysis of non-treated lesions demonstrated a limited self-healing of the induced defect that was statistically inferior than GGMA-rASC treatment. The self-repair ability of cartilage lesions, which is commonly reported in the rabbit model[42, 43] has been minimized by implementation of chondral defects with critical-size for which penetration and damage to the subchondral bone plate was avoided.

The GGMA formulation successfully supported *in vitro* chondrogenesis of both mature and progenitor cartilage-forming cells. In a rabbit model, controlled delivery of cells into chondral lesions was achieved, while adequate spatiotemporal crosslinking supported volumetric filling of cartilage lesions and in situ retention of cells. Following 8 weeks of treatment, the combination of GGMA-rASC, supported full thickness regeneration of critical size lesions, good integration / bonding with

native cartilage. Such combination therapy exhibited highly favorable physicochemical characteristics and good biological performance which may support less invasive and complex surgical procedures for cartilage repair.

ACKNOWLEDGEMENTS

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CHAPTER VI

MEDICAL DEVICE FOR DELIVERY OF THERAPEUTIC FORMULATIONS AND METHODS OF USE THEREOF.

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Provisional National Application PT109853

ABSTRACT

The present chapter describes the development of a device for delivery of injectable formulations into the articular joint, to an articular cartilage defect. The proposed device is intended to be used during an arthroscopy procedure to deliver liquid or semi-liquid formulations that comprise fluid vehicles, matrices, cells, therapeutic drugs, biomarkers, and biomolecules, or any related combinations into a cartilage defect. The device enables controlled and localized delivery of the formulation to a specific focal defect in articular cartilage by an arthroscopic procedure. This device enables the simple and efficient implantation of cartilage repair devices rendering a less intrusive and patient convenient procedure for treatment of articular cartilage lesions.

INTRODUCTION

Articular cartilage defects are present in the majority of patients undergoing arthroscopic procedures[1]. The high epidemiological prevalence of articular cartilage defects is related with the limited endogenous repair capacity of cartilage. Damaged cartilage leads to a fibrous tissue with inferior biological performance that will suffer constant and irreversible degeneration throughout life.

The aim of many currently available surgical procedures is to promote a more effective cartilaginous response by using marrow stimulating techniques, such as microfracture[2]. These procedures, generally considered first-line treatments for focal defects, are cost-effective and clinically useful because patients often have reduced pain and improved joint function. However, in most cases, these approaches render only a temporary effect, and their long-term therapeutic effect is questionable[3]. The fibrocartilage tissue formed, when compared with normal hyaline articular cartilage, has inferior mechanical and biochemical properties, is poorly organized, contains significant amounts of collagen type I, and is more susceptible to injury. The loading and breakdown of this repair tissue with time eventually leads to premature osteoarthritis.

The aim of currently available therapeutic procedures based on cell or tissue transplantation is to achieve regeneration of hyaline-like cartilage tissue. The autologous chondrocyte implantation (ACI) procedure, first introduced in 1987, has been the most widely used surgical procedure[4] . The first generation ACI involves the use of a periosteal flap or a collagen sheet which is fixed to the surrounding cartilage to create a reservoir for injection of a suspension of ex vivo expanded autologous chondrocytes[4, 5] . The clinical effectiveness for articular cartilage repair has been reported in several studies. However, ACI application may be inadequate in certain scenarios because of anatomic factors, and difficulty of fixation of the periosteal flap or collagen sheets to retain the chondrocyte suspension in the cartilage defect. Notwithstanding, ACI represents the only clinical available cell-based therapy for cartilage repair. However, even this therapy presents several performance drawbacks resulting in surgical complications, which normally leads to repetition of surgery in 25 to 36% of the ACI treated patients[6].

Improvements have aimed to overcome the intrinsic technical disadvantages of first generation ACI by using cartilage tissue engineering (TE) grafts developed with three-dimensional matrices that contain autologous chondrocytes (MACI - matrix-induced autologous chondrocyte implantation) for cartilage regeneration[7]. The limitations of current methods have justified the use of alternative

matrices in combination with therapeutic cells for cartilage repair. An attractive approach is the use of cell encapsulation platforms by which cells are delivered and retained at the focal cartilage defect site. In this regard, hydrogels present appealing properties for tissue engineering and regenerative medicine applications as they swell and retain large amounts of water, are tissue mimetic and can be delivered using minimally invasive procedures (i.e., injection)[8]. In addition, hydrogels provide a highly hydrated microenvironment (resembling extracellular matrix - ECM) and can be crosslinked *in situ* allowing cell encapsulation and preservation/induction of their differentiated phenotype. Ideally, an injectable hydrogel should also present a sol-gel transition mechanism suitable for clinical purposes, i.e., it should be liquid to facilitate homogeneous cell distribution and injection, while being capable of rapidly solidifying after implantation and adhere to the cartilage defect.

In spite of developments at research level, limited improvements have been made at clinical level regarding the surgical technique used to deliver these combination products into cartilage defects and assure retention within the lesion site. The procedures used to treat cartilage defects typically involve an arthrotomy or arthroscopy procedure[9]. Arthrotomy procedures imply longer surgical intervention duration and higher cost of the treatment. Arthroscopy procedure can reduce surgical cost and enable outpatient treatment. Arthroscopy procedures have been applied to the treatment of cartilage defects using MACI[10, 11]. Briefly, the MACI construct is inserted through a portal by means of a cannula or forceps and fixed using additional fixation means such as a bioabsorbable suture[10] or fibrin glue[11]. As the use of an expansion liquid can compromise integrity of MACI construct and impede its fixation, the inflow of expansion liquid is stopped causing collapse of the joint and impeding further its internal visualization. To avoid this, during the implantation procedure the expansion liquid is temporarily stopped, the articular cavity collapses to its normal position, and an expansion gas is used to expand again the articular cavity for implantation of the construct. Previous to the work described in this thesis, several other methods and devices have been developed which are intended to improve surgical efficacy of treatment methods by means of arthroscopic procedures. For example:

- Patent WO2005079881 describes an arthroscopic method for scaffold-free cell implantation in mammals, whereby the filling of a cartilage defect is made by a catheter injection of a cell suspension using a portal. In order to fix the cells into a defect, the cell suspension is allowed to settle under the influence of gravity. The adhesion of the cell suspension is assured by adjusting viscosity and concentration of divalent cations. However, this document does not provide

a technical solution for the above-mentioned problem, especially taking into consideration that the articular cavity becomes inflated by the expansion fluid that can easily remove the cell suspension from the defect site.

- Patent US20050137600 describes surgical instruments for preparation of an articular cartilage site for arthroscopic implantation of an articular cartilage repair device into subchondral bone.
- Patent US2007077236 describes a method wherein a cell suspension is applied through a portal into the articular joint by injection, which is followed by the sedimentation of cultured cells and adhesion of cells to the subchondral bone and/or cartilage. In this document, the media with cells is applied simultaneously with a support material during an arthroscopic procedure. The mixing of these components occurs during the flow displacement, and upon application, the support material coagulates. The proposed method claims the use of a sealant material over the coagulated cell/support. However, this method does not limit the application of cell suspension and support material to the defect site, neither impedes the migration of the cell suspension, the support material or the sealant material to the remainder of the articular cavity.
- Patent EP1656960 discloses the arthroscopic delivery of cells seeded in a foldable support matrix by means of an introducer featuring a telescopic working channel terminated by a cap. The device to be implanted is delivered by folding it conveniently through a channel. However, the application of this device is not trivial as the unfolding and fixation of the implantable device in the cavity raises manipulation challenges that contribute to extend the duration of the surgical procedure and increase likelihood of procedure variability by different surgeons. Taking into consideration the demanding requirements imposed by material characteristics, and biological requirements of cells being co-delivered to the defect site, the injection and fixation of a hydrogel-based formulation is not technically convenient using any of the devices.
- PatentWO2013155359 discloses a two-needle device featuring a cannula/needle and a longer inner needle, which can be used as a delivery system for stimulation of the bone chondral interphase. Although the system can be used to deliver certain therapeutic agents into the articular cavity, it would not be possible to use such system to address the challenge pertaining to delivery of a therapeutic formulation comprising for example, biomaterial matrices or therapeutic formulations consisting of combinations involving these.

- Patent US2012283833 also describes a method for diagnosing and treating articular related bone conditions. This method applies to conditions in which the underlying bone is physiologically impaired, which does not necessarily apply to all types of chondral defects.

The present chapter describes succinctly the development of a device for delivery of therapeutic formulations to the articular joint and, more particularly to an articular cartilage defect in a human or animal subject.

MATERIALS AND METHODS

Design and Classification Constraints

The medical device proposed should address basic requirements related with its intended purpose and final device classification. From a medical device perspective, the proposed device will be used by penetrating inside the body through its surface in the context of a surgical operation to the joint, which renders a surgically invasive device of very limited localized effect. In addition, it is foreseen that the device will be used for short term duration, i.e. less than 60 minutes, which defines its use as transient. These aspects are important to define the classification of the device and defining conformity assessment procedures applicable to the approval of the device. It is outside the scope of this chapter to define thoroughly all the applicable requirements applicable to the design, manufacturing and CE marking of the device. So, the main design criteria are following summarized.

The basic design concept departs from a set of principles, namely:

- Small diameter device, as to allow insertion through limited cross section ports during arthroscopy;
- Expandable geometry, as to provide coverage of cartilage lesions when juxtaposed to articular surface;
- Multi-channel, as to provide several options for delivery of formulations, and/or extraction of fluid, and/or transmission of radiation, or even visualization purposes.

In Addition, the adopted design should be simple in order to reduce both the total number of components and overall cost of manufacturing. Taking in account the limited area for insertion and the need to cover the surface of cartilage, In addition, the device should be serializable and should meet basic safety requirements related with invasiveness and direct tissue contact by using

materials normally adopted with devices of similar use, if available, or which are normally adopted in devices of similar classification.

Therefore, materials and manufacturing methods to be used in the several device components should be able to meet safety requirements and correspond to methods normally adopted for the selected materials. From a concept perspective, the device could be either manufactured for single use or multi use (reusable), as long appropriate reutilization procedures are carried out. In this regard, the repeated use of sterilization methods, such as ethylene oxide (ETO) or autoclave, may need to be validated in order to demonstrate that there is no detectable loss of characteristics or properties using established analytical methods. Taking into account the envisaged properties, it is anticipated that the device may be manufactured from a variety of materials meeting the regulatory requirements applicable to medical devices. The delivery arm may be composed by a thermoplastic material, such as polyolefin, polyester, or polyvinyl chloride. The flexible arm may be composed by a thermoplastic elastomer (TPE) selected from TPA-polyamide TPE; TPC-copolyester; TPO-Olefinic TPE, TPS-Styrenic TPE; or TPV-thermo plastic rubber. The delivery arm can be also composed by a thermoset material selected from siloxane, or polyorganosiloxane, polysiloxane. The cup may also be composed by a TPE selected from TPA-Polyamide TPE; TPC- copolyester TPE; TPO-olefenic TPE; TPS-styrenic TPE; TPU-urethane TPE; or TPV- thermoplastic rubber. Lateratively the suction cupmay be composed bya a thermoset material selected from siloxane, polyorganosiloxane or polysiloxane.

Device Design

A device was designed to be used during an arthroscopy procedure. It main function is to deliver a therapeutic formulation into a cartilage defect by an arthroscopic port as to provide a continuous connection between the cartilage defect cavity and an aseptically controlled environment outside the body. Several design iterations were performed in order to finalize a device concept, first by paper drawing and lastly by using a Computer Assisted Design (CAD) software, SolidWorks (2016, version 9000). For a better understanding of the device and its main components, the device is composed by several parts and sections (see Figures 1,2,3,4 and 5), which are following listed:

1. External sleeve;
2. Internal sleeve
3. Delivery arm;
4. Cup;

5. Interior surface;
6. Exterior surface;
7. Internal channel;
8. Plunger;
9. Connection tube;
10. Connection ports;
11. Double connector

Manufacturing and Assembling

A prototype of the device was manufactured using materials suitable to medical device manufacturing. The external sleeve was manufactured using polyetherimide (PEI) thermoplastic with an outer radius of 10 mm, 0.6 mm of wall thickness and 140 mm of length. The internal sleeve was manufactured using polyetherimide (PEI) thermoplastic with an outer radius of 8 mm, 0.3 mm of wall thickness and 160 mm of length. Both the external and internal sleeves were machined to the final dimensions by turning from a ULTEM® rod 1000 series (Sabic). The delivery arm and cup were manufactured from polysiloxane materials (Silastic, Dupont) by moulding using a machined master part. The dimensions of the delivery arm were an outer radius of 7 mm and 200 mm of length. The delivery arm featured 2 channels with 2 mm of diameter.

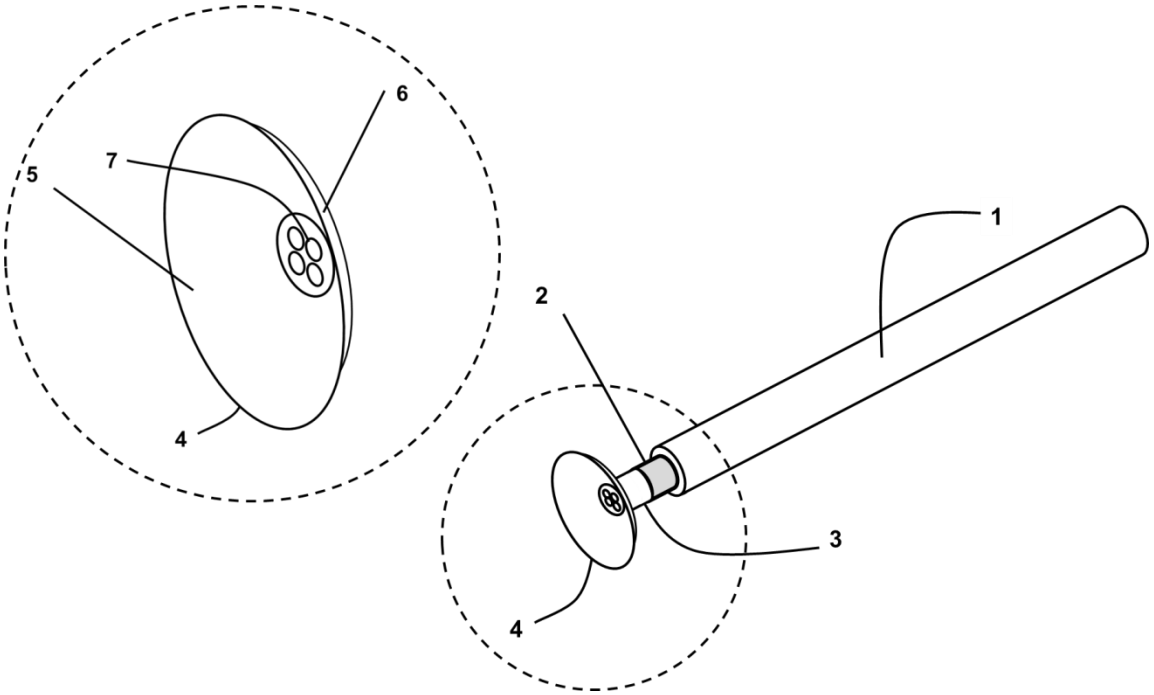

Figure 1 - *Schematic representation of the device for arthroscopic delivery.*
1) *External sleeve;* ***2)*** *Internal sleeve;* ***3)*** *Delivery arm;* ***4)*** *Cup;* ***5)*** *Interior surface;* ***6)*** *Exterior surface;* ***7)*** *Internal channel.*

Cleaning and Sterilization

After manufacturing, the parts were washed with a mild washing detergent (Softaskin, B.Braun), rinsed in ultrapure water (Milli-Q, Millipore), wrapped for sterilization and autoclaved (132 °C, 210 kPa, 50 min). After sterilization, all sterilized parts were unpacked inside a flow chamber and manually assembled according to the description previously provided. Following assembly, the device was wrapped and subjected to a new sterilization cycle by ethylene oxide (EtO) (80% EtO, 20% CO_2) at 45 °C ± 5 and 1400 mbar during 240 min.

Device Configuration

The device was used by adopting a configuration described in Figure 2, in which the device presents 2 connection tubes that are independently connected to distinct internal channels. Two syringes (one empty and one containing a therapeutic formulation) were connected by luer connectors to the connection tubes of the delivery arm. For that, a therapeutic formulation was prepared and inserted into a syringe and connected by a luer connector to the connection tube.

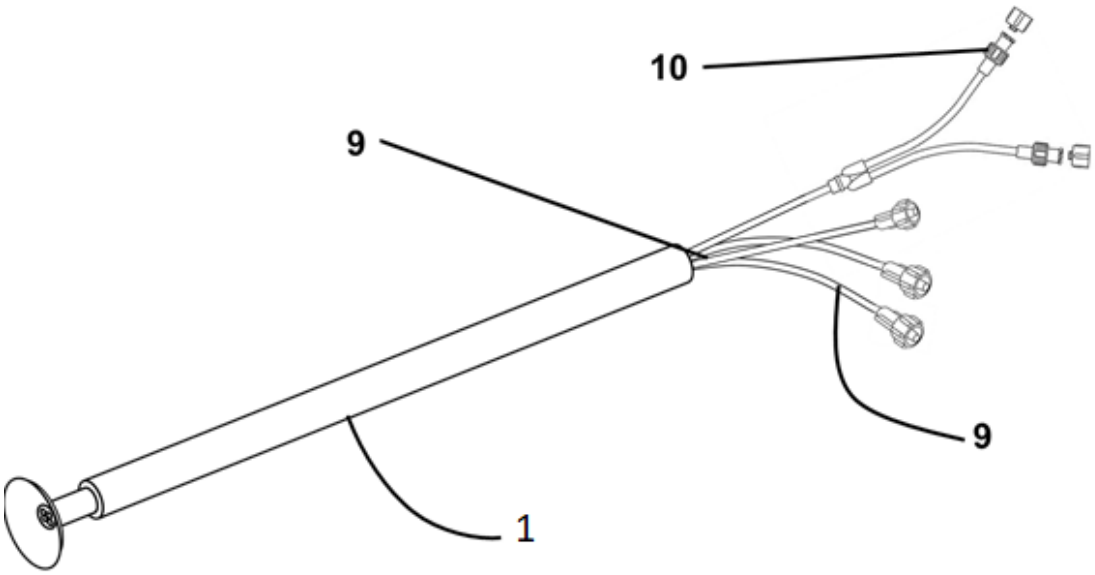

Figure 2 - *Schematic representation of the device for arthroscopic delivery featuring connection tubes and connection ports for delivery of therapeutic formulations, administration or removal of fluids, transmission of radiation, and visualization of treatment area.* ***1)*** *External sleeve;* ***9)*** *Connection tube;* ***10)*** *Connection ports;*

Knee Arthroscopy

A human cadaveric knee was used for demonstrating the efficacy of the device. The cadaveric knee was fixed in a support table. The knee was positioned in a 90° of flexion with the distal end of the leg in suspension. Two knee port were open with a surgical blade: one antero-lateral port and one antero-medial port. Execution of the procedure was carried out using arthroscopy instruments: an arthroscope tower fitted with wide angle telescope 30° with integrated light source (Karl Storz) connected to a monitor screen, and a double roller pump (Arthropump, Karl Storz) for articular cavity irrigation. Ringer's solution previously prepared (Sigma Aldrich) was used as fluid for irrigation of the cavity during the arthoscopic procedure. The intra-articular irrigation of the knee (100 mL/min) was performed during distension of the knee joint (average pressure 70 mmHg). The arthroscope was introduced in the knee cavity for intra-articular knee inspection. A 5 mm diameter defect was induced in the femoral condyle with a drill using a motorized drill.

Therapeutic Formulation

In order to assess the functional performance of the device, a therapeutic formulation analogue (total volume of 1 mL) comprised of methacrylated gellan gum hydrogel (2 % w/V) containing a suspension of $2x10^6$ cells/mL (human mesenchymal stem cells) was used. Phosphate buffer saline (Gibco) with calcium and magnesium was used as a fluid vehicle.

Results and Discussion

The wider adoption of a minimally invasive procedure such as arthroscopy to address cartilage defects benefits the treatment of patients, as it provides an operationally- simplified surgical procedure that enables reduction in surgical time and co-morbidity, which may ultimately support outpatient treatment regime and lower cost of treatment. Controlled spatiotemporal delivery of therapeutic formulations to the defect site is an important requirement, as it increases therapeutic efficacy and efficiency due to confined localized delivery to the defect site and avoids inadvertent loss of valuable therapeutic formulation to the remainder of the articular cavity resulting in no clinical benefit.

A significant challenge concerns the controlled spatiotemporal delivery of therapeutic formulations by a minimally invasive procedure such as arthroscopy, which is intended to treat cartilage defects during continuous expansion of the articular cavity without interruption. This problem is of special relevance taking into account that the arthroscopic procedure requires injection of an expansion

liquid or gas into the cavity space, which can negatively interfere with the therapeutic formulation being applied. Thus, any technical solution should limit the delivery of a therapeutic formulation exclusively to the defect site, impede its leakage to the remainder of the articular cavity and avoid the invasion of the cartilage defect site by the fluid used for articular cavity expansion. In addition, a technical solution should be compatible with the expansion medium, which may be either a liquid or gas, so as to avoid a double step articular cavity expansion procedure.

The magnitude of this challenge is increased for low viscosity therapeutic formulations, as these formulations may easily flow out from the defect site and spread throughout the remainder of the articular cavity during the implantation procedure.

Device

In order to address the challenge of delivering injectable formulations into the articular compartment, a device was designed and developed. Figure 1 presents a schematic diagram of the proposed device and its main components. The device is intended to be used during an arthroscopy procedure to deliver a therapeutic formulation into a cartilage defect by an arthroscopic port as to provide a continuous connection between the cartilage defect cavity and an aseptically controlled environment outside the body. This device and its components have a distal end and a proximal end. The distal end is intended to be used inside the articular cavity, while the proximal end faces the surgeon and provides access to the interior of the device. The device has been designed to exhibit enough flexibility to allow easy entering and maneuvering within the articular cavity, during the arthroscopy procedure.

The device is composed by a flexible external sleeve (1) that has the shape of a tube and comprises a lumen. The device comprises an internal sleeve (2) with the shape of a tube, which moves along its main axis within the external sleeve, to which is concentric. The device comprises a semi flexible delivery arm (3) that is concentric to the internal sleeve to which is solidary. At its distal end, the delivery arm features a conical flexible cup (4), which upon axial movement of the delivery arm towards its distal end, is exposed outside the external sleeve and allows for expansion of the cup to its normal shape – see Figure 3.

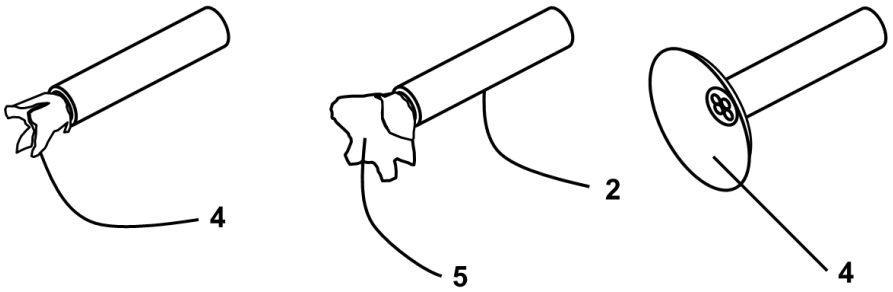

Figure 3 - *Schematic representation of the gradual expansion of the cup during axial displacement of the delivery arm towards the distal end within the external sleeve**. 2)** Internal sleeve; **4)** Cup; **5)** Interior surface;*

At its distal end, the cup can be placed juxtaposed to the cartilage defect, so as to cover the cartilage defect. The cup features interior (5) and exterior surfaces (6), which provide distinct contact surfaces within the articular cavity. The interior surface of the cup may be used to contact predominantly with cartilage surface, while the exterior surface may be used to contact with synovial and expansion fluids during the arthroscopy procedure. The cup exhibits flexibility to adjust its shape to the opposing cartilage surface that surrounds the defect. In one embodiment, the internal surface of the cup is placed in contact with cartilage as to assure a tight seal between cartilage surface and the cup and provide a confined volume between the cup and the cartilage defect that, by this way, is isolated from surrounding fluid contained in the articular cavity – see Figure 4.

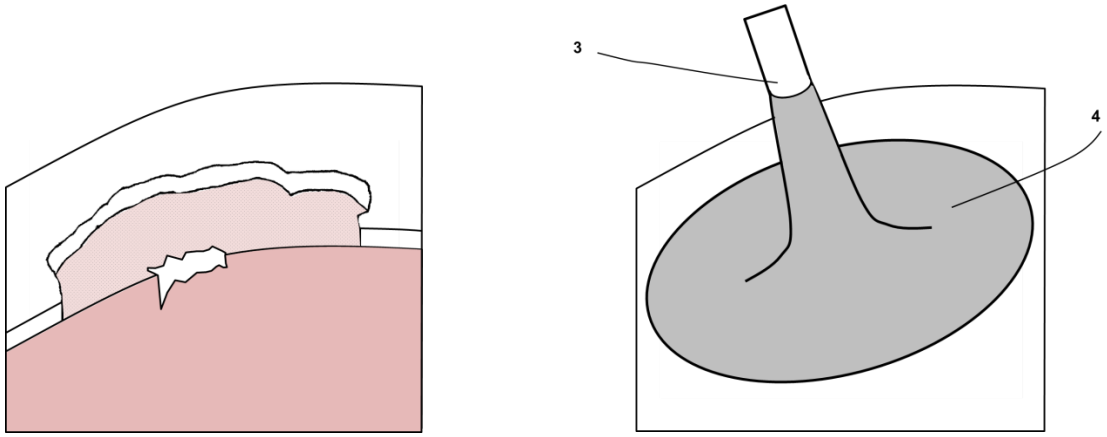

Figure 4 - *Schematic representation of a cross section of an articular cartilage defect, surrounding cartilage and: exposed subchondral bone and exposed subchondral bone with cavitation (right) and the cup covering an articular cartilage defect (15) – perspective view. **3)** Delivery arm; **4)** Cup;*

When the device is held against an opposing surface, such as articular cartilage surface, to which its main axis is orthogonal or oblique, the flexible cup can adapt itself to the opposing surface. In

one embodiment, the interior surface of the cup is concave as to provide a better adjustment of the cup to the opposing convex cartilage surface in the joint. In another embodiment, the interior surface of the cup is convex as to provide a better adjustment of the cup to the opposing concave cartilage surface in the joint. In one embodiment, the size of the cup can be selected according to the size of the defect. In an equally preferred embodiment, the geometry of the cup can be selected according to the concave or convex radius of the opposing cartilage surface.

The delivery arm has one or more internal channels (7) along its length that could be used for several purposes – see Figure 5. At their distal end, each internal channel terminates openly at the interior surface of the cup, and more preferably at the center of the cup. At least one internal channel is used for extraction of fluid from the articular cavity. In one embodiment, the extraction of fluid from the articular cavity by an internal channel is made by application of a negative pressure at the proximal end. The extraction of fluid is limited to the confined volume located between the cup and the articular defect site, creating a negative pressure within the confined volume. At least one internal channel is used for transporting a therapeutic formulation from the proximal to the distal end for delivering it into a cartilage defect. The displacement of the therapeutic formulation in the internal channel is assured by a positive pressure applied at the proximal end. The displacement of the therapeutic formulation is assured by a movable plunger (8) that moves coaxially inside the internal channel and impedes accumulation of the therapeutic formulation along its path. In another configuration, one internal channel could be used for transmission of light, such as visible, infra-red or ultra-violet light or other non-ionizing radiation such as radio waves or microwaves that could be used to stimulate or initiate physicochemical processes within the cartilage defect site. Another internal channel could be used for passing an optical fibre to visualize the articular cavity and more specifically the cartilage defect.

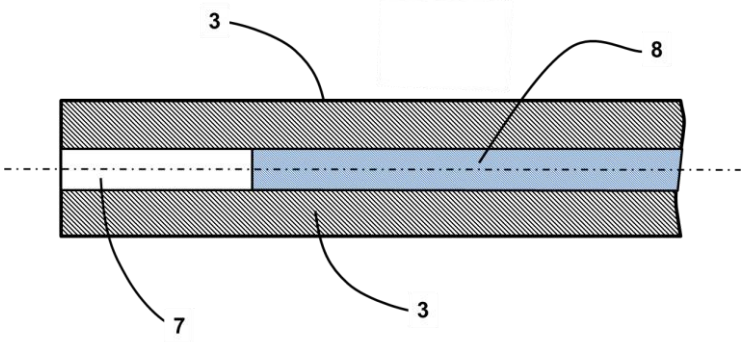

Figure 5 *- Schematic representation of the cross section of the delivery arm showing one channel and respective displaceable plunger.* ***3)*** *Delivery arm;* ***7)*** *Internal channel;* ***8)*** *Plunger;*

At its proximal section, the device features connection tubes (9) that are connected to the internal channels, which terminate in connection ports (10) compatible with standardized connectors, such as luer systems, or equivalent purpose connectors to be used multi-purposely. In a preferred embodiment, the device features luer connectors, including double connectors (11) – see again Figure 2. The connectors are used for connecting syringes (20) or other containers comprising therapeutic formulations. In another embodiment, the connection tubes are used for administration and/or removal of fluids. Such connection tubes and connectors could be used for transmission of light, other non-ionizing radiation, or used for visualization of articular cavity.

Method of Use

Using the device, a method for arthroscopic implantation of a given therapeutic formulations into an articular cartilage defect is possible to be established. A possible method of use can comprise the next steps:

1. Application of an expansion fluid to an articular cavity or surface containing the defect via arthroscopy;
2. Determination of the area, depth and location of the cartilage defect aimed to be repaired;
3. Introduction of the device by the arthroscopic port into the articular cavity;
4. Placement of the device juxtaposed to the cartilage defect site inside the articular cavity;
5. Displacement of the delivery arm with subsequent expansion of the cup inside the articular cavity;
6. Covering of the cartilage defect site by the expanded cup;
7. Sealing the defect site from surrounding articular environment by the cup, with concomitant creation of a confined volume at the cartilage defect site;
8. Optional removal of fluid from the confined volume underneath the cup by application of negative pressure.
9. Delivery of the therapeutic formulation by respective displacement into the confined volume.
10. Optional application of light or other radiation to the confined volume or therapeutic formulation by transmission of radiation.
11. Optional visualization of the cartilage defect site.

12. Maintenance of the cup at the cartilage defect site as to promote contact or fixation of the therapeutic formulation to the cartilage defect site, and to allow progress of any physicochemical reaction taking place in the therapeutic formulation.

13. Removal of the cup from the cartilage defect position with concomitant exposure of the cartilage defect site to the surrounding fluid.

14. Collapse of the cup by axial movement of the delivery arm towards its proximal end.

15. Removal of the device.

Knee Arthroscopic Results

Following the methods previously described, the device was successfully inserted in the articular cavity following the induction of the defect a human cadaveric knee. The device was inserted by the antero-medial port. Using image monitoring, the device was placed juxtaposed to the cartilage defect site inside the articular cavity – Figure 6a. The delivery arm was displaced as to allow expansion of the cup inside the articular cavity and assure covering of the defect site by the cup – Figure 6b.

The device was firmly positioned at the defect site as to provide sealing of the defect site from surrounding articular environment by the cup – Figure 6c.

Using the plungers of the empty syringe, about 0.2 mL of fluid was removed from the confined volume underneath the cup upon the negative pressure created by displacement of the plunger. By displacement of the plunger, the therapeutic formulation was delivered into the defect as to assure its respective filling. The cup was maintained on the top of the cartilage defect site during approximately 5 minutes as to allow sufficient time for crosslinking of the matrix by divalent cations provided by the fluid vehicle and fixation of the therapeutic formulation to the cartilage defect site. The cup was subsequently removed with exposure of the cartilage defect site to the surrounding fluid – Figure 6d. The cup was collapsed by axial movement of the delivery arm relatively to the external sleeve towards its proximal end, and the device removed – Figure 6e. Optical observation confirmed fixation of the therapeutic formulation within the cartilage defect during the arthroscopic procedure.

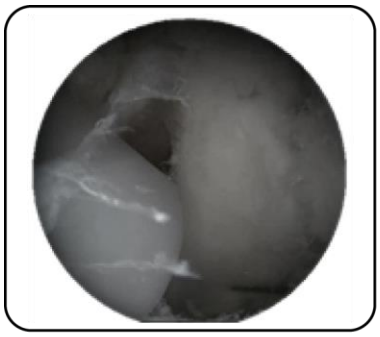

Figure 6a

Arthroscopic implantation of therapeutic formulations into an articular cartilage defect in a cadaveric model: device being juxtaposed to a cartilage defect

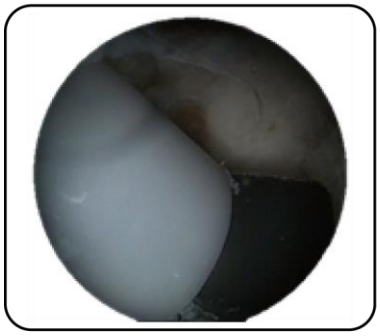

Figure 6b

Arthroscopic implantation of therapeutic formulations into an articular cartilage defect in a cadaveric model: expansion of the cup

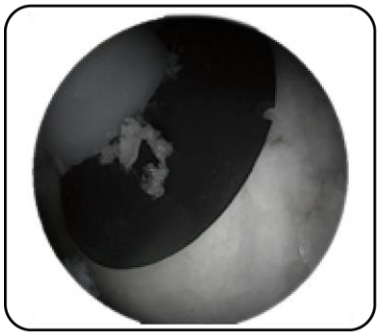

Figure 6c

Arthroscopic implantation of therapeutic formulations into an articular cartilage defect in a cadaveric model: covering of the cartilage defect by the expanded cup with subsequent delivery of a therapeutic formulation.

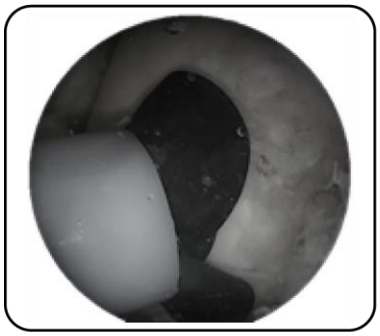

Figure 6d

Arthroscopic implantation of therapeutic formulations into an articular cartilage defect in a cadaveric model: removal of the cup.

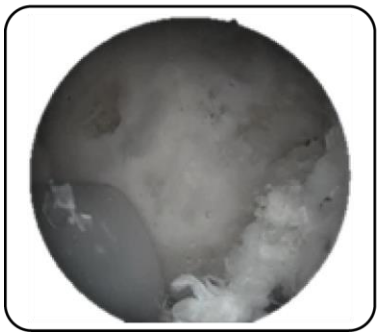

Figure 6e

Arthroscopic implantation of therapeutic formulations into an articular cartilage defect in a cadaveric model: therapeutic formulation delivered to the site.

Figure 6 – *Device in different stages of arthroscopic surgery*

Conclusions

A device for delivery of injectable formulations into the articular joint, in particular, to an articular cartilage defect was designed and developed. The device is intended to be used during an arthroscopy procedure to deliver a therapeutic formulation into a cartilage defect by an arthroscopic port as to provide a continuous connection between the cartilage defect cavity and an aseptically controlled environment outside the body. The proposed device is intended to deliver liquid or semi-liquid formulations, or any related combinations. Using the device, it was possible to deliver an injectable matrix encapsulating therapeutically relevant cells to a focal cartilage lesion during an arthroscopic procedure. It is anticipated that the therapeutic formulation may combine other different components such as fluid vehicles, matrices, cells, therapeutic drugs, biomarkers, and biomolecules. Using the testing formulation, the device enabled its controlled and localized delivery, providing a simple and efficient implantation procedure which is less intrusive and more patient convenient as compared to alternative arthrotomy procedures.

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SECTION IV

CHAPTER VII

Discussion, Future Perspectives and Conclusions

DISCUSSION

The clinical, social and economical relevance of cartilage lesions is well known and a real threat for the affected patient in terms of daily life and expectations for the future[1-9]. The cartilage lesion clinical diagnosis, in early phases of the disease, is difficult and pain is the most frequent symptom[1, 8]. Pain has a wide range of manifestations and, usually, presents a mechanical pattern, sometimes initiated by a previous trauma. Not uncommonly, the patient presents associated signs as swelling, hemarthrosis, block or giving-way[1, 8, 10]. Sometimes, cartilage lesion is an unexpected finding when other pathologies are diagnosed and/or treated[11]. Supplementary diagnosis tests are mandatory and MRI has a fundamental role as a complementary diagnostic examination for cartilage lesions[12, 13].

Cartilage lesions treatment is a very challenging issue with no definitive answers[7, 14, 15]. Stimulating bone marrow techniques like Microfracture (MFX) are widely used as a cartilage reparative procedure[8, 9, 16], given that it provides satisfactory short-term outcomes, at a very inexpensive cost. Results deterioration with time and the poor mechanical properties of the repaired tissue are major limitations[14, 17]. Osteochondral autograft transfer (OAT) in one or more plugs (mosaicplasty) are interesting cartilage restoration procedures, but the bond with native cartilage, the morbidity of the donor site, as well as the limited amount of harvested tissue are important drawbacks[7]. New approaches by tissue engineering techniques, using a temporary support to facilitate the growth of native or seeded cells are promising treatment alternative[14, 18]. The results seem to be auspicious[18]. Legal issues related with the use of cells, the short follow up, the higher cost and the frequent need for more than one surgery, are important limitations of such procedures[19]. Besides, more prospective, randomized, well designed and dimensioned trials are needed to achieve a conclusive answer regarding the real interest of such approaches[15, 19].

In our reviews, we found an overall agreement on what concerns the diagnosis and prognosis, but we failed to find a consensus on what concerns treatment of cartilage lesions[3, 7, 8, 14, 20-29]. Treatment results reported are confusing and none has clearly proven to produce a hyaline cartilage surface or to offer better clinical results [3, 7, 20-29]. Due to ethical issues, evaluation of the repair in clinical practice is mostly based by clinical evaluation and does not routinely include the histological or even macroscopic assessment by a second look arthroscopy[8]. This is a very important limitation regarding evaluation and comparison of different treatment options.

The Algorithm developed by International Cartilage Research Society (ICRS) can provide orientations for cartilage lesions' treatment and represents the current practice in this subject. So far, none of the current treatment techniques has proven unequivocally its superiority over the Microfracture (MFX) technique[8, 20, 21]. MFX has been used as the gold standard treatment to compare results between different treatments[21], but although the clinical symptomatic relief, MFX fail to restore hyaline cartilage[20, 29]. The repaired tissue achieved by MFX technique is mostly composed by fibrocartilage, has poor mechanical properties, and clinical results suffer deterioration with time[9, 20, 28, 29]. Alternative treatments are already available in the market, but for now, there is still limited clinical evidence to demonstrate their superiority[29].

Articular cartilage (AC) is derived from skeletal blastemal and is identified as early as the fifth embryonic week and is a special type of cartilage[30] composed mainly by water[30, 31] distributed in the interfibrillar space of the extracellular matrix (ECM) produced by sporadic chondrocytes[31, 32]. The ECM is composed by collagen (10-20%), glycosaminoglycans (GAGs) and proteoglycans (PGs) [31-33]. Oligosaccharides, monosaccharides, glycoproteins and non-collagen proteins are other constituent parts of the ECM[31-33]. Collagen II is almost the only type of collagen found in AC and can reach 90-95% of the total collagen present[31]. Collagen type X and XI are detected in a minimum amount and type V, VI and IX collagen are also residually detected [31]. ECM assures diffusion of the nutrients and the great amount of the ECM water component allied with the very specific cartilage structure which is divided in distinct layers or zones corresponding to distinct functions, render cartilage excellent viscoelastic properties and high resistance to shear and compressive forces, while protect sub-chondral plate and bone[30, 31, 33]. The macroscopic appearance of the repaired tissue and chemical properties, namely the relative amount of collagen type II, found in the repaired tissue are important indicators of cartilage quality and efficacy of cartilage restoration procedure[34]. These intrinsic characteristics are ultimate goals to achieve when developing cartilage repair methods[34].

A significant work has been performed to repair cartilage with hydrogels[33, 35]. Their adoption opens the possibility of implementing a minimally invasive surgery and reducing morbidity to the patient[35].

Nevertheless, reported studies are difficult to compare, because of the wide diversity of methodologies, evaluation criteria, techniques, animal models, materials and cells involved.

Most of all, most reported comparison studies have been performed between treatment groups and empty lesions. Only few studies compare the treatment group with a reference treatment[36-

38]. Thus, conclusions achieved for those studies, have a limited significance for clinical practice. Other limitations have been pointed out: relative animal immaturity and inappropriateness of animal model [39-43], inadequate dimension of the sample[36, 44-50], short follow up[39, 41, 44, 48, 50-58], lack of biomechanical evaluation[40, 41, 47, 53, 58-62], specific limitations of the design study[52, 63-66], poor representativeness of human pathology[67], origin-cell identification not possible in the majority of the studies, experimentation under a load bearing condition distinct to humans[36, 53] absence of specific rehabilitation program[55]. Further studies in this area should address as much as possible these limitations, by providing realistic and well-designed studies.

Our *in vivo* study allowed the performance evaluation of GGMA+rASC treatment for potential clinical practice, by comparing the respective repaired tissue with the repaired tissue obtained by MFX treatment, the gold standard treatment. On other hand, due to the fact that in our study the sub-chondral bone was not intentionally disturbed, and sub-chondral plate respected, migration of endogenous cells to lesion site is very limited, making the repaired tissue the result of the implanted GGMA+rASC.

The rabbit is a validated animal model widely used in cartilage repair studies[68-70]. Rabbits are easily available, do not need complicated handling, cage or care and present a better cost-effectiveness when compared with other animal models[71]. However, the very thin cartilage thickness and small dimensions of the cartilage surface in condyles and trochlear groove of the rabbit[71], are limitations for translational studies with humans where the cartilage surface and average thickness differ significantly[72]. Besides, the relative position of the knee in rabbits is more horizontal when compared with humans, which determines a partial weight-bearing position, creating more mechanical differences[73]. Another limitation of the rabbit animal model is the possibility of spontaneous repair with cartilage healing for cartilage lesions under 3 mm [74, 75]. This is especially true in young immature rabbits[74, 75].

Due to those limitations pointed above, the rabbit has important limitations for translational human studies, yet it remains as a very valuable model for screening of the potential of new treatments and strategies in early stages of product development [68-70, 72]. As such, and to maximize the available chondral surface, in our study, 4 mm diameter chondral lesions were created, so that a critical - size defect was object of study, minimizing confounding with intrinsic spontaneous healing.

In most of the studies revised, macroscopic and histological evaluation was analyzed more frequently by the O'Driscoll score, followed by the Wakitani score. In addition, other scores such

as ICRS, Pineda, Mankin, Moran, Wayne, Sellers, Capplan and Susante have been also used to assess cartilage quality. We must highlight that none of these scores were validated by biochemical analysis. The only score validated by biochemical analysis is the Bern score. However, Bern score is more suitable for tissue-engineered constructs rather than *in vivo* repaired tissues. In our study, three scores were used simultaneously to assess cartilage quality and outcome: O'Driscoll, Pineda and Wakitani. The selection of such scores was based on the comprehensive analysis published by Rutgers et al. [34], where, Pineda and O'Driscoll are suggested as most adequate for scoring *in vivo* repaired animal cartilage, providing both a simple and comprehensive analysis, respectively. The broader numerical range used in the O'Driscoll system has been reported to increase the likelihood of finding statistically significant differences, which occurred in fact, in our study.

The Wakitani score is a modification of the Pineda score, developed to particularly study cartilage repair in rabbits [34]. It includes additional categories such as 'surface regularity', 'thickness of reparative cartilage compared with surrounding cartilage' and 'integration of donor cartilage with adjacent cartilage'. Given such pertinence for our study, this score was also used, although only the Pineda score has been validated [34].

Gellan gum (GG) is a natural, anionic bacterial-derived linear exopolysaccharide discovered by Kelco in 1978[76]. Composed of a tetrasaccharide repeating sequence of two β-D-glucose, one β-D-glucuronic acid and one α-L-rhamnose is produced by a fermentation process of the Sphingomonas elodea bacteria. GG has been initially exploited in food and pharmaceutical applications[76] and recently studied for its potential for cartilage repair[38, 70, 77, 78]. GG can form a three – dimensional network allowing cell encapsulation, diffusion of nutrients and metabolic products ensuring cell viability. The physic-chemical properties of GG formulations allows its injection directly into the lesion providing volumetric filling of an irregularly shaped lesion. Such characteristics are attractive towards accomplishing a less invasive surgical procedure. Some limitations of GG are known, including: week mechanical properties, and a high temperature window for solubilizaton (90°C-100°C) and gelification (50°C)[76]. Methacrylated Gellan Gum (GGMA) formulation, compared with others GG formulations has better solubility at physiological temperature and improved spaciotemporal crosslinking, facilitating implantation procedure during arthroscopic surgery. GGMA presents increased storage modulus as compared to the unmodified polysaccharide, 89.5 ± 7.4 kPa and 56.2 ± 1.4 kPa, respectively[79]. Matrix stiffness, due to the increased concentration or biomechanical cues, seems to benefit cell viability and chondrogenesis[80].

Previous *in vivo* studies have been conducted to repair cartilage using Gellan Gum. Oliveira et al.[81], performed and treated chondral lesions perforating the subchondral bone, and obtained very positive outcomes. Under the work of this thesis, it was considered beneficial to avoid unnecessary damaging of healthy subchondral bone to treat a focal cartilage lesion. According to the ICRS score, most of the cartilage lesions found in current clinical practice are grade 3 whish are found in about 55% of all patients submitted to an arthroscopy. Only in 5% of patients a grade 4 ICRS lesion have been found[82, 83]. Thus, in the majority of cartilage lesions found, it is desired to avoid an invasive treatment procedure which includes unnecessary damage of the healthy sub-chondral. Therefore, scaffolds offering a good adhesion to the sub-chondral bone plate without need of additional fixation and avoiding damage the sub-chondral bone seems to be an interesting approach for cartilage repair study.

Regarding the use of cells, in our review it was found that chondrocytes were the first choice for cell-seeded scaffolds. Chondrocytes are the natural residents of cartilage, therefore an autologous treatment approach shall in fact yield the optimum outcomes. Nevertheless, limitations on what regards dedifferentiation during cell expansion, need for double surgery in most treatment approaches (a first intervention for cartilage biopsy and a second for treatment), lead to a solution which is inherently lengthy in time and inevitably expensive. Alternatives have been extensively studied, in order to achieve a commercially more attractive solution. Mesenchymal stromal/ stem cells (MSC) are mostly targeted due to their immunopriveliged nature and proven potential to differentiate into the chondral lineage, opening strong avenues for off-the-shelf allogeneic therapies. The scalability of cell production results in a less expensive therapy as compared to chondrocytes. MSC originating from synovial (sMSC), muscle (mMSC), bone marrow (bmMSC) or adipose tissue (aMSC / ASC) were explored in the context of cartilage repair. According to the revised bibliography, it was stated that sMSC and bmMSC show a greater chondrogenic potential when compared with to aMSC or mMSC[84-86], and that sMSC also present a greater proliferation potential [84-86]. Although some authors did not find better results in cartilage regeneration when MSC were used[63, 87-89], others reported a better cartilage and bone formation and superior cartilage bonding to adjacent native cartilage[46, 90, 91].

In our study, we studied the potential of mesenchymal stem cells derived from adipose tissue for encapsulation in GGMA hydrogel after isolation and expansion. Adipose tissue is a tempting tissue source for collecting stem cells. The potential great amount of graft, the easy and non-invasive way to collect adipose tissue and high MSC yields are interesting advantages of this option. Our results

show the trilineage differentiation capacity for CD73+/CD90+/CD105+/CD31-/CD34-/CD45- adipose stromal/ stem cells (ASC), with expressive mineralization, lipid formation and GAG deposition upon osteogenesis, adipogenesis and chondrogenesis, respectively. Such ASC were used for cell encapsulation studies within GG and GGMA hydrogels to evaluate cell viability and chondrogenesis. At a very first assessment, distinct metabolic activity was observed among GG and GGMA formulations, with advantage for GGMA 1% and 2% w/V as compared to GG at 1% w/V. After 7 days of culture, GGMA 2% w/V revealed highest metabolic activity by encapsulated ASC. On what regards chondrogenesis, it was found that, by 3 weeks of culture, ASC ($5x10^6$/mL) upregulated collagen type II expression to a similar extent when cultured within both GGMA 2% w/V and GG 1% w/V. It is possible that longer periods of culture could reveal a differential behavior, once cells reach a more differentiated state. For instance, primary chondrocytes cultured within same hydrogel formulations, expressed significantly higher collagen II when cultured within GGMA 2% w/V as compared to GG 1% w/V.

A more thorough analysis on what regards hydrogel mechanical properties; porosity, pore size and pore interconnectivity and also matrix architecture, would allow a better understanding on the physical factors that are influencing cell behavior, on what regards both viability and functionality. Nevertheless, the set of analytics performed allow to confirm that such hydrogel formulations are favorable to sustain ASC and chondrocytes metabolically active for at least up to 3 weeks *in vitro*, while providing a favorable environment for chondrogenesis: hyaline chondrocytes maintain a healthy ECM profile (upregulation of collagen II concomitant with downregulation of collagen type I), and ASC show a positive trend towards chondrogenesis by up-regulating gene expression of collagen type II, at a ratio not inferior than collagen type I. Based on these set of experiments, GGMA 2% w/V formulation was selected for more dedicated performance studies both *in vitro* and *in vivo*. It was found that, ASC cultured at a higher density ($10x10^6$/mL) within GGMA 2% w/V, significantly upregulated gene expression of collagen type II as compared to collagen I, while also synthesizing superior collagen II protein indicating that, as well-reported by literature, cell-cell contact, and interaction plays a pivotal role on biological outcomes, particularly chondrogenesis. In this experiment, ECM was more thoroughly examined through staining of samples, whereas intense safranin O/ fast green for identification of the cartilage matrix, alcian blue for detection of sulphated GAG and IHC of human collagen type II, demonstrates a healthy chondrogenic ECM. The absence of collagen type I deposition also indicates development of non-fibrous cartilaginous

tissue. These findings supported the interest of the GGMA 2% w/V hydrogel for delivery and retention of chondrogenic cells in lesion site for cartilage repair.

For *in vivo* studies, excellent tolerability was also demonstrated. No abnormalities of the synovium, signs of inflammation, loose bodies, osteophytes or degenerative process were found. The defects treated with the GGMA+rASC combination showed compact bright tissue filling, despite macroscopic variability observed between defects. The margin between the surrounding cartilage and the defect was less evident in GGMA+rASC construct than in non-treated control group. MFX and the control non-treated group (empty) presented an irregular filling of the lesion site, with tissue of a dim appearance, sub-chondral overgrowth and reduced collagen type II staining at the top layer of the tissue. The poor quality of the repaired tissue found in the defects treated with MFX treatment, can eventually lead to treatment failure upon recurrence of symptoms[16, 92] and disfavor adequate load bearing as well as smooth, pain-free joint motion[19, 20, 68].

We could demonstrate that GGMA+rASC can treat chondral lesions and promote the restoration of cartilage thickness, integration / bonding with native cartilage, as well as intense and reasonably homogenous staining of ECM throughout the lesion site. Quantitative assessment of repair by all three scoring systems indicates significant improvement in cartilage repair as compared to the untreated lesions. According to the O'Driscoll scale, GGMA+rASC additionally over performed the gold-standard MFX (Fig.5). Those findings demonstrated the interest of the GGMA-ASC combination, inspiring new studies, strategies and approaches for cartilage repair.

This study was conducted using the GGMA 2% w/V hydrogel without any fixation system to retain within the chondral lesion site or the requirement of a scaffolding structure shaping, which could be an advantage as compared to other cell-based and tissue engineered cartilage products currently in clinical development[93]. In addition, viscous and sol-gel transition properties of the tested GGMA 2% w/V hydrogel allowed controlled delivery of the matrix containing autologous ASC [68], directly to lesion site, which favored delivery and retention of cells *in situ*. This fact is of significant importance as cell retention at lesion site is one of the main indicators of success for lesion repair[93, 94].

In order to achieve optimal delivery and retention of the hydrogel+cells composition to lesion site through a standard wet arthroscopy, a dedicated surgical device was developed. When the device is introduced in a joint through an arthroscopic portal, a terminal suction cup is expanded from the inner sleeve and placed over the lesion. The aspiration of the articular arthroscopic fluid under the suction cup allows vacuum-like conditions that permits the adhesion of its borders to the cartilage

surface, creating the conditions for the administration of the treatment agent in the right place, without any dilution with the arthroscopic articular fluid, maintaining the correct concentration, during the time necessary for polymerization and fixation. Other important advantage of this surgical instrument is avoiding an extended surgical incision, and its underlying inconveniences including increased morbidity. This device proved its functionality in delivering the GGMA hydrogel into an articular cartilage lesion during an arthroscopy conducted in a cadaveric experiment. The design of such surgical tool allows its compatibility for controlled delivery of virtually any liquid, viscous or malleable substance into desired locations of the articular surface. To the best of our knowledge, no similar device is commercially available which leads to an exciting opportunity for professionals working in the arthroscopic field.

FUTURE PERSPECTIVES

The cartilage lesion studied in this work, as well as in most experimental studies reported, is an acute defect. Nevertheless, the majority of cartilage lesions treated in a current clinical practice are sub-acute or chronic. It is understandable that mechanical, inflammatory and specific conditions are not the same in acute or chronic lesions, therefore the reparative process may differ as well. Thus, it seems desirable to conduct studies comparing differences in acute and chronic lesions and comparing the treatment response to those conditions.

Another question to be addressed relates to the quality of treatments with time. As reported elsewhere, results of cartilage repair using current practices tend to deteriorate with time given suboptimal repair tissue developed. Therefore, long-term studies are needed to provide sound results to sustain development of a product which shall transform current clinical practice for cartilage repair.

As exposed above, the animal model used in this study is appropriate for initial and screening studies, yet to obtain relevant performance outcomes, new experiments need to be conducted in a large animal model to enable treatment defects, similar to those found in human conditions.

This screening study concerned morphological and histological evaluation of repaired tissue. The authors acknowledge that mechanical evaluation of the regenerated tissue, as well as a more in-depth sub-chondral morphologic evaluation shall be further performed. Image assessments,

namely through micro-computed tomography and/or optical coherence tomography techniques, are valuable for a better evaluation of the repaired tissue, towards validation of such innovative therapies.

Additional approaches for cartilage repair management may include the use of MFX technique associated with the GGMA hydrogel as a patch, like in the so-called MFX AMIC technique. Herein, the perforations in the chondral plate and through the sub-chondral bone allow the migration of the host cells into lesion site. By applying GGMA hydrogel over the lesion, as a patch, it could retain such host cells in lesion site during the time necessary to grow new cartilage. This option may well be an effective, technically simple and inexpensive option for smaller diameter lesions.

Despite intensive research in the field of cartilage repair, fundamental questions still await for answers, apportion of which could become available if experiments are conducted in a more standardized way, allowing better comparisons in between different studies.

CONCLUSIONS

The work developed under the scope of this thesis allowed the delivery of new insights on what regards pros and cons of current clinical practice for treatment of cartilage lesions, as well as R&D efforts to provide better solutions for this growing unmet clinical need.

As part of a tissue engineering & regenerative medicine strategy for repair of cartilage lesions, hydrogel biomaterials were extensively reviewed as promising scaffolding systems given their highly hydrated nature. In fact, hyaline-like cartilage development was proven in a considerable number of *in vivo* studies, providing confidence on this approach. A list of learnings was appointed during this review, on what are the benefits and limitations of the developed technologies and surgical approaches, towards better design of the proposed GGMA-based hydrogel solution. For instance, the majority of the published papers addressed small, acute and a full-thickness cartilage defect in a non-weight bearing area, conditions that are very different from those found in human patients which is a concerning limitation considering translation of experimental learnings towards human treatment. A potential advantage of using hydrogels for cartilage repair is its suitability for arthroscopic delivery, yet, in many studies, hydrogel properties did not seem compatible with this minimally invasive approach. These aspects were highly considered during design of experimental studies as well as during development of the proposed hydrogel biomaterial.

Herein, the methacrylated gellan gum (GGMA) formulation was optimized towards achieving optimal features, including:

i. Dissolution compatible with surgical setting: GGMA 2% w/V is fully dissolved in deionized water in less thaan 30 minutes under mild agitation at room temperature.
ii. Cells, within an ionic solution, are easily added to the GGMA solution and gently mixed, achieving a homogeneous mixture within seconds;
iii. The hydrogel+cells mixture yields a user friendly viscous solution, compatible with required working time both *in vitro* and *in vivo* – full reticulation occurs within 5-10 minutes.
iv. The viscosity of the solution allows spatial control for delivery within chondral lesion site, permitting a 3D volumetric filling, without spillovers.
v. Gelification occurs by ionic crosslinking, using solely physiologic solutions, avoiding need of additional instruments or non-physiologic reagents.

vi. Spatiotemporal control of hydrogel crosslinking allows hydrogel+cells retention within lesion site
vii. No additional fixation systems were required

Such GGMA 2% w/V formulation successfully supported *in vitro* chondrogenesis of both mature and progenitor cartilage-forming cells, including chondrocytes and adipose derived mesenchymal stromal/stem cells (ASC), respectively. Chondrogenesis was evaluated by assessing collagen types I and II gene expression by qRT-PCR, and also deposition of cartilage extracellular matrix, assessed by safranin O, alcian blue and immunohistochemistry of collagens type I and II. Better understanding of physical and mechanical properties of the system would be desired for future experiments.

In a rabbit model, controlled delivery of autologous ASC at 10 M/mL into chondral lesions was achieved, while adequate spatiotemporal crosslinking supported volumetric filling of cartilage lesions and *in situ* retention of cells. Following 8 weeks of treatment, the combination of GGMA-rASC, supported full thickness regeneration of 4 mm diameter critical size lesions, including good integration and bonding with native cartilage, superior than gold standard treatment microfracture, according to the well-established O'Driscoll scoring system. It was concluded that such combination therapy exhibited highly favorable physicochemical characteristics and good biological performance, which may support less invasive and complex surgical procedures for cartilage repair.

In what regards less invasive surgical procedures, in order to complete the proposed therapeutic system, a device for delivery of injectable formulations into the articular joint, in particular, to an articular cartilage defect was designed and developed. The device is intended to be used during an arthroscopy procedure to deliver a therapeutic formulation into a cartilage defect by an arthroscopic port as to provide a continuous connection between the cartilage defect cavity and an aseptically controlled environment outside the body. Using the device, it was possible to deliver the developed GGMA 2% w/V formulation to a focal cartilage lesion created in a cadaveric model, during an arthroscopic procedure. The device enabled its controlled and localized delivery, providing a simple and efficient implantation procedure which is less intrusive and more patient convenient as compared to alternative arthrotomy procedures. It is anticipated that the therapeutic formulation may combine different components such as fluid vehicles, matrices, cells, therapeutic drugs, biomarkers, and biomolecules.

The work developed in this thesis delivers promising outcomes on what regards an innovative treatment approach for treatment of critical size focal articular cartilage lesions. Such approach includes a methacrylated gellan gum hydrogel at 2% w/V, combined with adipose derived mesenchymal stromal/stem cells at $10x10^6$ /mL, which yield physicochemical properties compatible for controlled delivery into lesion site, arthroscopically through the developed dedicated surgical tool.

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ANNEX I

ANNEX I

Authorship and Co-Authorship of scientific papers, publications, oral communications, posters and patents, in the field of cartilage repair:

Publications

Bilayered Silk/Silk-NanoCaP Scaffolds for Osteochondral Tissue Engineering: In Vitro and In Vivo Assessment of Biological Performance

Yan L. P., Silva-Correia J., Oliveira M. B., Vilela C. A., Pereira H., Sousa R. A., Mano J. F., Oliveira A. L., Oliveira J. M., and Reis R. L.

Acta Biomaterialia, vol. 12, isseu 2015, pp. 227-241

Doi:10.1016/j.actbio.2014.10.021, 2014

Emerging Concepts in Treating Cartilage, Osteochondral Defects, and Osteoarthritis of the Knee and Ankle

Pereira, Hélder, Ibrahim Fatih Cengiz, Carlos Vilela, Pedro L. Ripoll, João Espregueira-Mendes, J. Miguel Oliveira, Rui L. Reis, and C. Niek van Dijk.

Osteochondral Tissue Engineering, pp. 25-62. Springer, Cham, 2018.

Doi: 10.1007/978-3-319-76735-2_2

Conference abstract - ISI Web of Sceince Indexed

Development & performance assessment of a new ATMP for cartilage tissue engineering

da Silva Morais A., Correia C., Vilela C. A., Gertrudes A., Learmonth D., Oliveira J. M., Sousa R. A., and Reis R. L.

Frontiers in Bioengineering and Biotechnology

Doi:10.3389/conf.FBIOE.2016.01.02807, 2016

Silk bilayer scaffolds can induce fast integration with subchondral bone and support cartilage repair.

Yan L. P., Vilela C. A., Pereira H., Sousa R. A., Oliveira A. L., Oliveira J. M., and Reis R. L.

Journal of Tissue Engineering and Regenerative Medicine,

vol. 8, issue sup1, pp. 409, 2014

Comunication Poster

Stem Cells from the adipose tissue as allogenic player in cartilage repair: in vivo performance.

Correia C., da Silva Morais A., Vilela C. A., Gertrudes A., Santos T. C., Oliveira J. M., Espregueira-Mendes J. D., Sousa R. A., and Reis R. L.

World Stem Cell Summi, 2014

Comunication - oral

Cartilagem: lesões e tratamento

Vilela CA, Rui L. Reis, Espregueira-Mendes, JD

II Encontro Nacional dos Serviços de Ortopedia – GECA, 2012

ACI, MACI ou MASI? Indicações em 2015

Vilela CA, Rui L. Reis, Espregueira-Mendes, JD

XII Congresso da SPAT- SPAT, 2015

A one-step combined therapy for cartilage repair: development and performance assessment''

da Silva Morais A., Correia C., Vilela C. A., Gertrudes A., Santos T. C., Oliveira J. M., Espregueira-Mendes J. D., Sousa R. A., and Reis R. L.

EU Meeting -2014, vol. 8, pp. 205-205

Doi:10.1002/term.1931, 2014

Patent

Viscosupplement composition comprising ulvan for treating arthritis,

Sousa R. A., Gertrudes A., Correia C., da Silva Morais A., Gonçalves C., Radhouani H., Vilela C. A., Santos T. C., Oliveira J. M., Espregueira-Mendes J. D., and Reis R. L.,

WO/2015/174872, 2015

Products for repairing Cartilage lesions, Method of preparation and uses thereof

Reis R. L., Sousa R. A., Correia C., Vilela C. A., da Silva Morais A., Gertrudes A., Oliveira J. M., Santos T. C., and Espregueira-Mendes J. D.

Nº: 107642 H, 2014

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Título da Tese:

"Cartilage Repair: the role of a scaffold in the repair of a cartilage lesion"

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Professor Doutor Rui L. Reis

DOUTORAMENTO EM MEDICINA

Ano de conclusão: 2018

Universidade do Minho, 01 de agosto de 2018

Assinatura: